Activation of Mitochondrial Apoptosis and Regulation of Ceramide Signalling by COX-2 Inhibitors in Colon Cancer

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Abstract
Bcl-2 family of proteins is implicated in the malignant tumors including colorectal cancer. Activation of Bcl-2 inhibits the pro-apoptotic proteins (Bax and Bad) and regulates many biological processes such as apoptosis, cell proliferation and cell growth. As the mitochondrial enzymes are involved in sphingolipid metabolism, it can regulate ceramide formation and in turn mitochondria play a central role for the regulation of ceramide induced apoptosis. Bcl-2 Bcl-xL activates sphingosine kinases (SKs), resulting in the accumulation of S1P (sphingosine-1-phosphate), thereby reducing apoptosis. In the present study, the anti-neoplastic effects have been observed of Etoricoxib and Celecoxib, two COX-2 selective non-steroidal anti-inflammatory drugs (NSAIDs), and Diclofenac, a preferential COX-2 inhibitor NSAIDs, in the early stage of colon cancer in rats. These NSAIDs regress the expressions of Bcl-2 and SK-1 and promote apoptosis. Gross morphological analysis revealed the occurrence of raised mucosal lesions called MPL or multiple plaque lesions, which were maximum in the 1, 2-Dimethylhydrazine (DMH) treated group and their number repressed with the co-administration of the NSAIDs. An abnormal histo-architecture like hyperplasia and dysplasia were evident in the carcinogenic group, which were reduced with NSAIDs co-administration.

Keywords: Apoptosis; Bcl-2; Colorectal cancer; DMH; NSAIDs; SK-1

Introduction
Cells die in response to various stimuli and during apoptosis or programmed cell death this happens in a controlled way to maintain homeostasis in constantly replicating tissues such as the colon [1]. The pro-apoptotic Bax and Bad protein form important components of Bcl-2 family which is one of the dominant member in the mitochondrial form of apoptosis [2, 3]. It also involves down-modulation of Bax-antagonists such as Bcl-2 [4]. Translocation of pro-apoptotic Bax and Bad from the cytoplasm to the mitochondria is a crucial event in apoptosis cascades and therefore Bax has been recognized as an important mediator of anticancer drug-induced cell death [5]. Cytochrome c interacts with Apat-1 and triggers the formation of apoptosome protein complex in the cytosol and in the presence of ATP and Apat-1 then becomes an allosteric activator of the caspase cascade and the proteolytic demolition of the cell [6, 7]. Caspases are the family of endoproteases, it provides critical links in cellular regulatory networks to control inflammation and cell death. Activation of apoptotic caspases results in either the inactivation or activation of the substrates, and the production of a cascade of signalling events permitting to the controlled demolition of cellular components [8]. Stimulation of inflammatory caspases promotes the production of active pro-inflammatory cytokines and promotes apoptosis. Caspases are critical links as their dysregulation underlies human diseases which includes inflammatory disorders and cancer [9].

Cell signalling pathway mediated by ceramide has been shown to contribute to terminal cell differentiation, cell cycle arrest and apoptosis [10] as well as to cell proliferation [11]. Ceramide may modify the relationship between pro-apoptotic (i.e., Bax and Bad) and anti-apoptotic (i.e., Bcl-2 and Bcl-XL) members of the Bcl-2 family of proteins. Key components of the sphingolipid metabolic pathway, are the ceramide and its metabolites, which includes sphingosine and sphingosine-1-phosphate (S1P). S1P has been exhibited to stimulate cell growth and prevent apoptosis [12]. Sphingosine kinase (SK) catalyses the phosphorylation of sphingosine that results in the synthesis of S1P. Sphingosine kinase (SK1) can be stimulated by a variety of growth factors, cytokines and mitogens [13].

Presently, in an experimental colorectal cancer (CRC) in rodents, apoptotic proteins and sphingosine kinase have been targeted in the COX-2 inhibitors mediated cancer cell killings. Three COX-2 inhibitors used on comparable basis are 1) Celecoxib, a methylphenyl trifluoromethyl pyrazolyl benzene sulfonamide, 2) Etoricoxib, a chloromethyl methylsulfonyl phenyl bipyriridine and 3) Diclofenac, a dichloro anilinophenyl acetic acid.

Materials and Methods

Chemicals

1, 2-Dimethylhydrazine (DMH) and Bradford reagent have been purchased from Sigma Aldrich (St. Louis, MO, USA). Celecoxib and Diclofenac were a generous gift from Ranbaxy Pharmaceuticals (Gurgaon, India). The primary antibody against Bcl-2, Bax, Bad, Apat-1, Cyt c, Caspase-9, Caspase-3, SK-1 and β-actin was purchased from Santa Cruz Biotechnology, Inc., CA (USA). Alkaline phosphatase conjugated secondary antibodies and BCIP-NBT were purchased from Genet, Bangalore, India. All other chemicals and reagents used in the present study were of analytical grade and purchased from the reputed Indian manufacturers.

Animal Procurement
Female Sprague-Dawley rats of body weight between 100-150g were obtained from the inbred population of the Central Animal House, Panjab University, Chandigarh. The animals were acclimatized for at least 1 week and given normal diet (rodent chow) and water ad libitum. They were maintained as per the principles and guidelines of the Ethics Committee of Animal Care of Panjab University and in general, followed the NIH guidelines (Rule No. 23-85, as revised in 1985). They

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Received September 21, 2015; Accepted October 21, 2015; Published November 02, 2015

Citation: Sanyal SN, Jain S, Ghanghas P, Rana C (2015) Activation of Mitochondrial Apoptosis and Regulation of Ceramide Signalling by COX-2 Inhibitors in Colon Cancer. Transl Med 5: 159. doi:10.4172/2161-1025.1000159

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were housed three/four per cage in polypropylene cages with a wire mesh top and kept in a hygienic bed of husk (regularly changed) in a well-ventilated animal room. The animals were also maintained at the ambient temperature and humidity, and under a 12 hr photoperiod of light and darkness, respectively. They were provided with a room cooler and room heater in the summer and winter months, respectively.

**Treatment Schedule**

Animals were assorted into the following groups

- **Group 1 (control):** Animals received food (rat chow) and water *ad libitum* daily.
- **Group 2 (Vehicle Treated):** Animals were administered the vehicle (1 mM EDTA–saline) subcutaneously (s.c.) in weekly injection and 0.5% carboxymethyl cellulose (CMC) sodium salt per oral (p.o.) daily.
- **Group 3 (1, 2-Dimethylhydrazine dihydrochloride (DMH)):** Animals were administered with DMH weekly at a dose of 30 mg/kg body weight (s.c.). The dose of DMH in early carcinogenesis has been established in our laboratory earlier [14]. DMH was freshly prepared in 1 mM EDTA–saline and pH adjusted to 7.0 using dilute NaOH solution.
- **Group 4 (DMH + Celecoxib):** celecoxib was given daily (p.o.) within its therapeutic anti-inflammatory dose (ED₅₀ for rats, 6 mg/kg body weight) along with the weekly administration of 30 mg/kg body weight of DMH [15].
- **Group 5 (DMH + Etoricoxib):** Etoricoxib was given daily (p.o.) within its therapeutic anti-inflammatory dose (ED₅₀ for rats, 0.6 mg/kg body weight) along with the weekly administration of 30 mg/kg body weight of DMH.
- **Group 6 (DMH + Diclofenac):** Diclofenac was given daily (p.o.) within its therapeutic anti-inflammatory dose (ED₅₀ for rats, 8 mg/kg body weight) along with the weekly administration of 30 mg/kg body weight of DMH [16].
- **Group 7 (Celecoxib):** Animals received only celecoxib (6 mg/kg) dissolved in 0.5% CMC per oral daily.
- **Group 8 (Etoricoxib):** Animals received only etoricoxib (0.6 mg/kg) dissolved in 0.5% CMC per oral daily.
- **Group 9 (Diclofenac):** Animals received only diclofenac (8 mg/kg) dissolved in 0.5% CMC per oral daily.

Four animals were taken in each treatment group. Body weight of the animals was recorded weekly till the termination of the treatment. After 6 week of treatment schedule, the animals were kept on overnight fasting with drinking water *ad libitum* and sacrificed the next day under an over anesthesia with ether.

**Gross Morphological Observation**

The colonic pieces were divided into proximal, middle and distal regions and immediately fixed in 10% buffered formalin for 24 hrs. The tissues were dehydrated in ascending series of alcohol (30-90%) and kept in 1:1 mixture of absolute alcohol and benzene for 1 hr. For embedding the tissues in wax, they were kept in benzene for 40-45 min and transferred sequentially to 1:1 benzene and wax mixture at 60°C for 1hr and then pure wax for 6 hour at 60°C with two changes. The tissues were embedded in wax and set in the wooden blocks, then five micron sections were cut using a hand driven microtome, stretched in warm water bath (58°C) and transferred to egg albumin coated slides. Slides were dewaxed in xylene, then hydrated in descending series of alcohol, stained with haematoxylin and dipped in ammonia water till blue colour is developed. In case of being over stained the slides are dipped in acid water. The slides were dehydrated in ascending series of alcohol and brought to 70% alcohol and then stained with eosin and differentiated in 90% alcohol. Slides were dipped in absolute alcohol, cleared in xylene and finally mounted in DPX, and viewed under a light microscope and photographed at 200X with a Carl Zeiss, Axioscope AI microscope to which was attached a Digital Camera (Jenoptix) [18].

**Isolation of Colonocytes**

Colonic epithelial cells (colonocytes) were obtained from the freshly isolated colons by the method of Mouille et al [19] as described earlier [14,16]. Trypan blue dye exclusion was performed each time for every group of isolated colonocytes and the viability of the cells observed ~90%.

**Western Blot Analysis**

Cell lysate preparation and western blots were performed as described earlier [20]. Protein samples (50 μg) from each treatment group were separated by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis. The separated proteins were electrohoresitically transferred to nitrocellulose membrane. Immunoblot was prepared using primary antibodies (Bcl-2: 1:1,000, β-actin: 1:1,000) from Santa Cruz Biotechnology, Inc., (USA) and alkaline phosphatase conjugated respective IgG secondary antibody at a dilution of 1:10,000 (Genei, Bangalore, India). A BCIP-NBT detection system was used to develop the blots. Bands obtained were densitometrically analysed using Image J software (NIH, Bethesda, Maryland, USA), and the density was expressed as grey values in densitometric units. β-actin has been used in the blots as the protein loading control. For the preparation of protein extracts, colon samples were removed at the end of the 6th week treatment period and rinsed. Total lysates were prepared in fresh ice-cold protein lysin buffer. The extracts were cleared of nuclei and residual mucosa by centrifugation at 10,000xg at 4°C. The supernatant was collected, and protein concentration determined by the method of Bradford [21].

**Immunofluorescence**

Immunofluorescence analysis was done as described earlier with primary antibodies (Bcl-2: 1:1,000, Bax: 1:1,000, Bad: 1:1,000, Apaf-1: 1:1,000, Cyt c: 1:1,000, Caspase-9: 1:1,000, Caspase-3: 1:1,000 and SK-1: 1:1,000), then incubated with respective FITC-conjugated secondary antibody and observed under a fluorescence microscope (AxioscopeAI, Carl Zeiss, Germany).

Immunofluorescence scores were calculated as follows [22]

IF score = % of positive cells X Intensity of fluorescence

Where, <10% positive cells = 0, 11-50 % positive cells = 2, 51-100% positive cells = 4, No staining = 0, Light Staining = 1, Moderate Staining = 2 and Bright Staining = 4

**Apoptotic Studies**

**Acridine orange/ ethidium bromide co-staining**

Fluorescence microscopy using the DNA binding fluorescent dyes, Acridine orange and Ethidium bromide was employed to study the
morbidity of the isolated colonocytes undergoing apoptosis. Briefly, 10 µl of the cell suspension (10^6 cells/ml) was mixed in PBS (pH 7.4) containing Acidine orange (1 µg/ml) and Ethidium bromide (1 µg/ml) [23]. Of this mixture, 10 µl was placed on a clean glass slide, covered with coverslip, and a minimum of 300 cells were counted (X200), using a fluorescence microscope (Axioscope A1, Carl Zeiss, Germany). The index of apoptosis was calculated as the percentage of the total number of cells with apoptotic nuclei to the total number of cells counted.

**Terminal deoxynucleotidyl transferase (TdT) dUTP Nick End Labelling (TUNEL) Assay**

Cells undergoing apoptosis were detected in paraffin embedded tissue sections (5 µm thick) by the TUNEL method, using TUNEL apoptosis detecting kit (GenScript, NJ, USA) according to the manufacturer’s protocol. A minimum of 200 cells per field were counted in four randomly selected fields and the percentage of apoptosis calculated.

**Statistical Analysis**

For analyzing the data, one way analysis of variance (ANOVA) was performed using statistical software package ‘SPSS v 16 for Windows’. The post-hoc comparisons of means from different groups were made by the Duncan’s test, where the preliminary analysis of variance indicated significant treatment effects. This is a multi-parametric test. a,b,c,d, means in each segment not sharing a common superscript letter differed significantly while the means sharing the same superscript did not differ significantly. Differences between the means were considered significant at p<0.01.

**Results and Discussion**

**Morphological analysis of colons**

Among all the nine groups, DMH alone showed higher number of MPLs, with a significant appearance of MPLs in all the three regions of colon: proximal, middle and distal, as shown in Figure 1. MPLs were recognized as either raised or non-raised lesions with identifiable tissue growth in carcinogen treated animals. MPL often appearing singly, but sometimes in multiples throughout the length of the colon. Appearance of MPLs at an early stage of neoplasia suggests that MPLs are the macroscopic sites for tumor growth. The control and vehicle treated animals from 6 week study show no such carcinogenic changes. In the Celecoxib, Etoricoxib and Diclofenac co-administered groups, MPLs were small and few in number as compared to DMH group, suggesting that they may inhibit tumor growth in possibly what could be the initiation stage of neoplasm. Celecoxib, Etoricoxib and Diclofenac when given alone show a minimum number of MPLs.

**Histopathological Examination**

After 6 week of treatment, colonic tissues were examined histopathologically by Haematoxylin and Eosin (H&E) staining where the intact cylindrical shape and typical open lumen architecture of crypts were seen in the control group (Figure 1). Also, there is a cellular relationship between crypt and stromal interspace. However, in DMH group, the interrelationship between crypt architecture and stromal tissues have largely been distorted with the presence of dysplasia and enlarged nuclei. The connective tissue was disoriented due to the dysplastic crypts. Also, high grade hyperplasia can be seen as characterized by increased number of deeply stained nuclei due to the presence of highly proliferative cells which were oval in shape and most frequently lost the polarity. Simultaneous administration with Celecoxib, Etoricoxib and Diclofenac largely corrected these carcinogenic changes. However, mild grade hyperplasia can still be evident. There were less dysplastic changes in DMH + Celecoxib, DMH + Etoricoxib and DMH + Diclofenac as visible in the connective tissue layer and less number of mitotic cells. As shown in Figure 2, the sections from vehicle treated and NSAID alone groups were showing normal crypt structure similar to those of the control group. Results from the present study showed the occurrence of invasive carcinomas leading to the muscularis mucosa both in tumors and in flat, non-raised mucosa of the colon. Most of the features of DMH-induced adenocarcinomas of the rat colon in the present study are similar to those of human colon carcinoma [24]. Invading crypts associated with the lymph nodes as observed in the present study were also similar to those...
reported in humans. Co-administration of Celecoxib, Etoricoxib and Diclofenac significantly inhibited the incidence of adenocarcinomas, total carcinomas as well as the total number of macroscopic lesions. Thus, results of this study establish that the present dose of DMH and NSAIDs (Celecoxib, Etoricoxib and Diclofenac), and the given time schedule of the doses is adequate enough to establish the experimental model of human colon carcinogenesis and its chemoprevention in rat.

**Activation of Mitochondrial Apoptosis**

Inhibition of mitochondrial apoptosis is common in cancers and have a role in various cellular functions including, cell growth, differentiation, development and apoptosis [25]. Initiation of mitochondrial-based apoptosis with the use of NSAIDs supports their chemoprevention and anti-cancerous potential [14,16]. The protein expression as observed by western immunoblot analysis shows that administration of DMH leads to an up-regulation of Bcl-2 (arrows), the anti-apoptotic mitochondrial membrane guard proteins, as compared to control while NSAIDs co-administration had significantly reduced it upto the basal level (Figures 3a and 3b). Immunofluorescence further confirms the higher amount of translated Bcl-2 in DMH group as compared to Control whereas DMH + Celecoxib, DMH + Etoricoxib and DMH + Diclofenac were having lesser Bcl-2 levels as compared to DMH group, as shown in (Figures 3c and 3d). Along with the upregulation of Bcl-2 levels, the expressions of Bad and Bax were downregulated in DMH group as compared to control (Figures 4a-c). Celecoxib, Etoricoxib and Diclofenac administrations were able to upregulate the Bad and Bax expression, and thus triggered the mitochondrial pathway of apoptosis in colon cancer. Vehicle treated or alone NSAID groups (Celecoxib, Etoricoxib and Diclofenac) also showed significant expression of Bax, Bad and a low expression of Bcl-2.

The immunofluorescence localization of Cyt c and Apaf-1 (arrows) in NSAIDs co-administration group is higher as compared to DMH alone group, as shown in Figure 5a-c. With the elevated cytoplasmic expression and localization of Cyt c and Apaf-1 in NSAIDs co-

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**Figure 4:** Immunofluorescence analysis Bax and Bad. 
- **a.** Expression of Bax in the colonic tissue sections (arrows), analyzed by immunofluorescence in colonic tissue sections of 6 weeks treatment schedule (X400). DMH + Celecoxib, DMH + Etoricoxib and DMH + Diclofenac group showing increased localization of Bax (arrows) as compared to DMH alone group. 
- **b.** Expression of Bax in the colonic tissue sections (arrows), analyzed by immunofluorescence in colonic tissue sections of 6 weeks treatment schedule (X400). DMH + Celecoxib, DMH + Etoricoxib and DMH + Diclofenac group showing increased localization of Bax (arrows), whereas DMH group is showing decreased expression of Bax. 
- **c.** Shows the immunofluorescent staining score of Bax and Bax. (\(\text{Immunofluorescent staining score}_{Bax}\) and \(\text{Immunofluorescent staining score}_{Bad}\)) represents in each segment not sharing a common superscript letter that differed significantly (Duncan’s test: \(p < 0.01\)).

**Figure 5:** Expression of Apaf-1 and Cyt-c analysed by immunofluorescence. 
- **a.** Expression of Apaf-1 in the colonic tissue sections (arrows), analyzed by immunofluorescence. DMH + Celecoxib, DMH + Etoricoxib and DMH + Diclofenac groups showing increased localization of Apaf-1 (arrows), whereas expression is decreased in DMH alone group. 
- **b.** Immunofluorescence analysis of Cyt-c for its expression and localization studies in colonic tissue sections of 6 weeks treatment schedule (X400). DMH + Celecoxib, DMH + Etoricoxib and DMH + Diclofenac group showing increased localization of Cyt-c (arrows) as compared to DMH alone group. 
- **c.** Shows the immunofluorescent staining score of Apaf-1 and Cyt-c. (\(\text{Immunofluorescent staining score}_{Apaf-1}\) and \(\text{Immunofluorescent staining score}_{Cyt-c}\)) represents in each segment not sharing a common superscript letter that differed significantly (Duncan’s test: \(p < 0.01\)).
Regulation of Ceramide/ S1P pathway

To further elucidate the role of Bcl-2 in promoting cell survival and blocking apoptosis in colon carcinogenesis, the expression of sphingosine kinase (SK-1) protein was checked. In the present study, the expression of SK-1 is higher in DMH alone group as compared to control, vehicle treated and alone NSAID groups (Figure 7a & b). The elevated expression of SK-1 is significantly reduced in the NSAIDs co-administered groups (Figure 7b). The expression and localization studies in colonic tissue sections of 6 weeks treatment schedule (X400). The DMH group is showing decreased expression of Caspase-3 as compared to control, whereas NSAIDs co-administered groups showing increased localization of Caspase-3 (Figure 8). The results from the TUNEL assay showed that the colonic sections of control, vehicle treated and alone NSAID groups detected significant level of apoptosis (Figures 8a and 9b). The number of TUNEL positive cells was high in these groups as compared to DMH. DMH treated group also showed a considerable decrease in TUNEL positive cells and an increase in the number of apoptotic cells after celecoxib, etoricoxic and diclofenac co-administration.

Conclusion

Ceramide induces apoptosis, while sphingosine-1 phosphate (S1P) functions as a survival factor in colon cancer. Mitochondria play a central role in ceramide induced apoptosis and regulated by Bcl-2 family of proteins. Presently, a high level of SK-1 which is responsible for the accumulation of S1P had been seen in DMH group and corrected by the protective role of sphingolipids against colon carcinogenesis may be the result of the conversion of complex sphingolipids to bioactive metabolites including sphingoid bases (sphingosine and sphinganine) and ceramide, which inhibit proliferation and induce apoptosis (programmed cell death) in various types of cancer cell [29,30]. Celecoxib, etoricoxib and diclofenac co-administration was able to inhibit DMH induced elevation of SK1 and in turn promotes apoptosis.

Regulation of Apoptosis by NSAIDs

The acridine orange and ethidium bromide co-staining of isolated colonocytes showed that the NSAIDs co-administered groups; DMH + Celecoxib, DMH + Etoricoxib and DMH + Diclofenac had more apoptotic cells with respect to the DMH alone group, as shown in Figure 8a-e. Viable cells glow green with acridine orange whereas the apoptotic cells were characterized by yellow colour with ethidium bromide. Apoptotic cell number in the control, vehicle treated and alone NSAID groups i.e., Celecoxib, Etoricoxib and Diclofenac was significantly higher in comparison to NSAIDs co-administration groups and DMH alone (Figures 8a-e).

The number of apoptotic cells undergoing apoptosis was significant and the intensity of staining was high in these groups as compared to DMH. DMH treated group also showed a considerable decrease in TUNEL positive cells and an increase in the number of apoptotic cells after Celecoxib, Etoricoxib and Diclofenac co-administration.
NSAIDs. This may lead to a novel chemopreventive strategy targeting the Bcl-2 proteins, regulating the enzymes involved in the ceramide metabolism, and downregulating the pro-survival growth factors in colon carcinogenesis.

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Figure 9: Apoptotic studies in rat colon by TUNEL assay. a. Photomicrographs (X400) showing treated groups (arrows). b. Shows the percent apoptotic cells as analysed by TUNEL assay. The number of TUNEL positive cells were counted and represented graphically. The percentage of apoptotic cells is increasing after Celecoxib, Etoricoxib and Diclofenac administration, whereas less number of apoptotic cells are present in DMH group.

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