

Original Article

Cyclooxygenase-2 knockdown using retinoic acid chalcone (RAC), a promising therapeutic strategy for colon cancer

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Received February 4, 2015; Accepted May 10, 2015; Epub May 15, 2015; Published June 1, 2015

Abstract: Retinoic acid is an effective agent in the treatment of epithelial and hematological malignancies. The present study demonstrates that retinoic acid chalcone (RAC), an analogue of retinoic acid inhibits cell proliferation and induces apoptosis in HCT-15 and CT26.WT colon cancer cell lines. In HCT-15 cells the percentage of apoptotic cells increased from 32.4 ± 3 , 45.0 ± 3 to $72.6 \pm 5\%$ respectively at 10, 15 and 20 $\mu\text{g}/\text{mL}$ compared to 3.7% in control. Similarly in CT26.WT cells the percentage increased from 28.6 ± 3 , 41.2 ± 3 to $65.4 \pm 5\%$ on treatment with 10, 15 and 20 $\mu\text{g}/\text{mL}$ concentrations of RAC after 72 h compared to $2.9 \pm 1\%$ in control. Western blotting, fluorescence-activated cell sorting analysis and reverse transcription-PCR assays were used to investigate these effects. RAC inhibited the overexpression of COX-2, PGE2 and PGE2 receptor (EP1 and EP4) in the colon cancer cell lines. RAC mediated inhibition of cell growth and induction of apoptosis through COX-2 inhibition was also confirmed by treating the HCT-15 and CT26.WT colon cancer cells with COX-2 inhibitor, indomethacin and transfection of cells with COX-2 small interfering RNA. In nude mice with tumor xenografts, treatment with RAC-supplemented diet caused inhibition of COX-2, PGE2, and PGE2 receptors (EP1, EP3, and EP4) in tumors. Thus RAC can be a potential candidate for the treatment of colon cancer through the inhibition of COX-2 expression and subsequent inhibition of PGE2 and PGE2 receptors.

Keywords: Cell proliferation, indomethacin, transfection, apoptosis, inhibition

Introduction

Prostaglandins are formed from arachidonic acid and the process is catalysed by cyclooxygenase (COX) enzymes [1, 2]. Among two isoforms of the COX enzyme (COX-2, COX-1), COX-1 is involved in the maintenance of tissue homeostasis in all the tissues and protects the mucosa of the gastrointestinal tract against gastric damage. In most of the tissues COX-2 is expressed minimally but is overexpressed at the sites of inflammation which then promptly returns to the basal level. It is reported that in colon, breast, pancreatic, lung, and stomach cancers COX-2 is overexpressed [3-13]. Induction of COX-2 is considered to be the indication of the colorectal tumorigenesis as in the aberrant crypt foci [14]. There is 40-50% and 80-90% overexpression of COX-2 in premalig-

nant adenomas and colorectal carcinomas, respectively [15]. A null mutation of COX-2 markedly reduces the number and size of intestinal tumours in the APC^{A716} knockout mice [16].

In colon cancer, COX-2 catalyzed arachidonic acid oxidation generates prostaglandins and highly reactive by-products that may accelerate the carcinogenesis process. However, the exact mechanism of COX-2 mediated tumour formation is not fully understood. High concentrations of free arachidonic acid can promote apoptosis, independent of prostaglandin formation [17]. It is possible that the increased levels of COX-2 serve to lower the intracellular level of free arachidonic acid and thereby prevent apoptosis. Thus, COX-2 catalysis may be simply a process that depletes an apoptotic signal. Alternatively, products formed by the enzymatic

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action of COX-2, presumably one of the prostaglandins, alter cell growth, apoptosis, angiogenesis or other steps involved in tumourigenesis [18-20].

COX-1 and COX-2 induce angiogenesis and PGE2 inhibits programmed cell death by inducing expression of the Bcl-2 proto-oncogene [20]. PGE2 and other prostaglandins often elevate intracellular cyclic AMP concentrations which can suppress apoptosis. A physiological antagonist of COX-2, 15-hydroxyprostaglandin dehydrogenase (15-PGDH), has recently emerged as one of the key players down-regulated in colon cancer [21, 22]. Cyclooxygenase pathway regulates PGE2 levels by establishing equilibrium between its formation and conversion into 15-PGDH [23]. However in colorectal, breast, and lung carcinomas the equilibrium is shifted to its formation side, suggesting the importance of inhibiting COX-2 expression [21]. Thus present study was devised to investigate the effect of RAC on COX-2 expression and the underlying mechanism in the colon cancer.

Materials and methods

Cell culture

The HCT-15, LS 174T and CT26.WT colon cancer cell lines were obtained from American Type Culture Collection (ATCC). All the cell lines were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% FBS at 37°C in an atmosphere containing 5% CO₂.

Reagents and chemicals

Retinoic acid chalcone was obtained from Sigma-Aldrich (St. Louis, MO, USA). Antibodies specific for COX-2, EP1, EP2, EP3, EP4, their secondary antibodies and an EP4 agonist were purchased from Cell Signaling Technology, Inc. (Beverly, MA, USA). The PCR primers of known sequences of EP1, EP2, EP3, and EP4 were purchased from Invitrogen.

Treatment

The HCT-15, LS 174T and CT26.WT cells were plated in 6 cm dishes and grown in DMEM for 24 h. Different concentrations of RAC in 200 µl volume was added to each dish. The cells were then incubated for different intervals of time at room temperature.

MTT assay

The HCT-15, LS 174T and CT26.WT cells at a density of 2.5×10^5 per 100 µL were seeded onto 96well plates and incubated overnight. The cells were pre-treated with 5, 10, 15 or 20 µg/mL RAC for 36 h followed by addition of 20 µL MTT solution to each well (5 mg/mL). Incubation of plates for 4 h at 37°C in 5% CO₂ was followed by removal of supernatants and addition of 150 µL. Plates were then placed on an orbital shaker for 5 min and the absorbance was recorded using the EnSpire™ 2300 Multilabel Plate Reader (PerkinElmer, Inc., Waltham, MA, USA) at 595 nm.

Analysis of apoptotic cell death by flow cytometry

For determination of RAC-induced apoptosis of the human colon cancer cells flow cytometry using the Annexin V-conjugated Alexa Fluor488 (Alexa488) Apoptosis Detection kit was employed. The cells after overnight serum starvation were treated with a range of RAC concentrations for 36 h. The cells were harvested and washed in PBS. Then the cells were incubated with Alexa488 and propidium iodide in the dark. FACS Calibur instrument (BD Biosciences) equipped with the Cell Quest 3.3 software was used for analysis of stained cells.

Western blot analysis

RAC-treated colon cancer cells were washed twice in PBS. Then, Lysis buffer (50 mM Tris-HCl pH 7.4, 137 mM NaCl, 10% glycerol, 100 mM sodium vanadate, 1 mM PMSF, 10 mg/ml aprotinin, 10 mg/ml leupeptin, 1% NP-40, and 5 mM cocktail) 2 ml was added to the cells. BCA method was used to determine protein concentration. The protein were loaded and resolved by electrophoresis on a 10% polyacrylamide gel. The semi-dry method was used to transfer proteins onto a PVDF membrane which was then blocked with 5% non-fat dry milk overnight. After TBST washing, membrane was incubated for 2 h with primary antibodies and then washed again with TBST before incubation with secondary antibodies for 2 h. Then X-ray autoradiography was performed and the gray scale images were analysed. Relative COX-2 protein concentrations were determined by densitometry of the scanned radiography image with the Quantity One 1-D Analysis v.4.5.2 software

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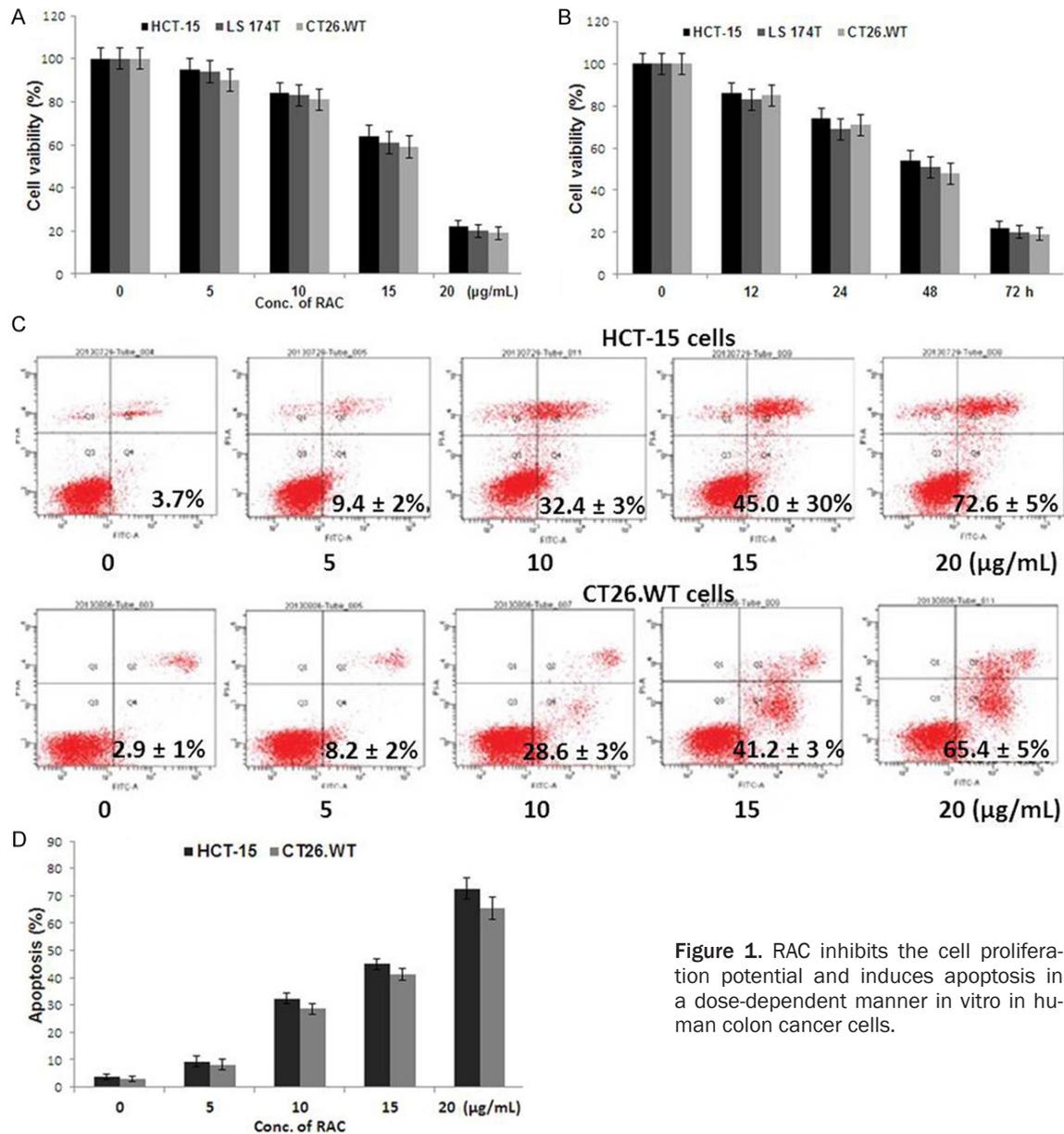


Figure 1. RAC inhibits the cell proliferation potential and induces apoptosis in a dose-dependent manner in vitro in human colon cancer cells.

(Bio-Rad). All values were normalized for loading in comparison with the appropriate β -actin signal.

COX-2-Small interfering RNA transfection of HCT-15 and CT26.WT cells

The HCT-15 and CT26.WT cells were transfected with COX-2 small interfering RNA (siRNA) using the siRNA Transfection Reagent kit (Santa Cruz Biotechnology, Inc.). The cells were grown to 70% confluency and then COX-2 siRNA was put on to the cells. After 24 h of transfection medium was replaced by fresh medium and the cells were incubated for an additional 48 h. The

cells were harvested and trypan blue exclusion assay was used for cell death analysis. Western blot analysis was used to examine the down-regulation of COX-2 expression.

Quantitative real-time reverse transcription-polymerase chain reaction (QRT-PCR)

Total RNA was isolated from the cells using Trizol (Invitrogen, Carlsbad, CA, USA) following the manufacturer's instructions. Total RNA was used for reverse transcription using DNA synthesis kit (Invitrogen). Primers for PCR were designed and PCR amplification of cDNA was performed at 35 cycles in a reaction mixture

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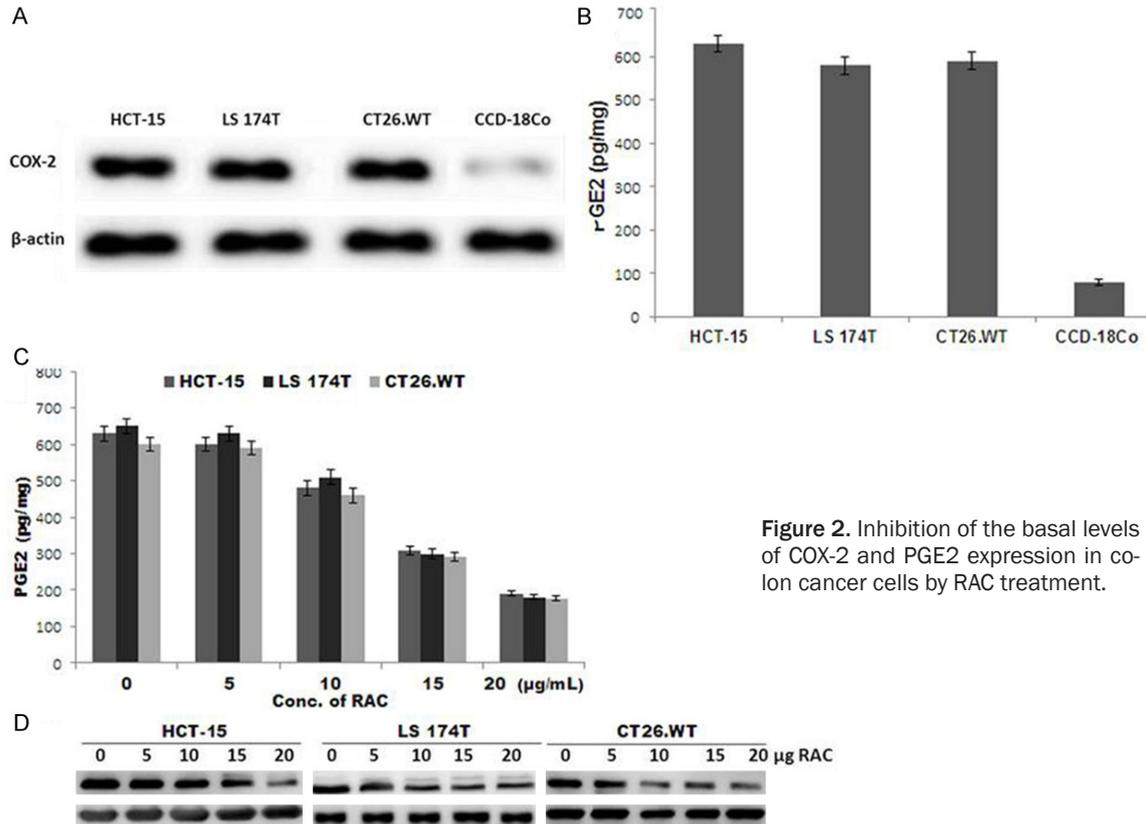


Figure 2. Inhibition of the basal levels of COX-2 and PGE2 expression in colon cancer cells by RAC treatment.

containing 10 μM Tris-HCl (pH 8.3), 1.5 μM MgCl₂, 50 μM KCl, 0.01% (w/v) gelatin, 200 μM dNTP, *SNP5*-specific primers (0.5 μM each), and 2.0 U of platinum *Taq* DNA polymerase (Invitrogen). For each reaction, two negative controls were performed consisting of omission of the RT step or omission of the target cDNA. Real-time results were collected and analyzed (Standard Curve Method) using the Sequence Detection System (SDS) software, version 2.0 (ABI), according to the manufacturer's protocol.

Animals and tumor xenograft assay

Athymic *nu/nu* 6-week-old female mice (Harlan Sprague-Dawley) were fed irradiated chow. The animals were divided into treatment group and control group with 10 each and in 0.2 mL Matrigel (Basement Membrane Matrix, High Concentration; BD Biosciences) 2×10^5 HCT-15 cells were injected. The treatment group of mice were injected with 20 $\mu\text{g}/\text{mL}$ of RAC on every third day whereas the control group received DMSO alone. Tumor growth was measured with a caliper and mean of the tumor volume at each point was normalized in each group to the mean volume measured at the first injection. The experiment was stopped after 52

days of treatment. Dissected tumors, after weighing were fixed in 10% formalin and embedded in paraffin wax. The formula, $(1 - \text{MT}/\text{MC}) \times 100$ was used for calculation of inhibition rate of tumor growth (MT & MC are mean of normalized tumor masses of treatment and control groups, respectively). All animals were maintained in specific pathogen-free conditions and all experiments followed the FELASA guidelines.

Statistical analysis

GraphPad Prism 5 (GraphPad Software, Inc., San Diego, CA, USA) was used for data analysis. The data is represented as the mean \pm standard deviation from triplicate experiments. Student's t-test was used for statistical differences assessment and $P < 0.05$ was considered statistically significant.

Results

Retinoic acid chalcone (RAC) inhibits proliferation of human colon cancer cells

The results from MTT assay showed a significant inhibition of proliferation of HCT-15, LS

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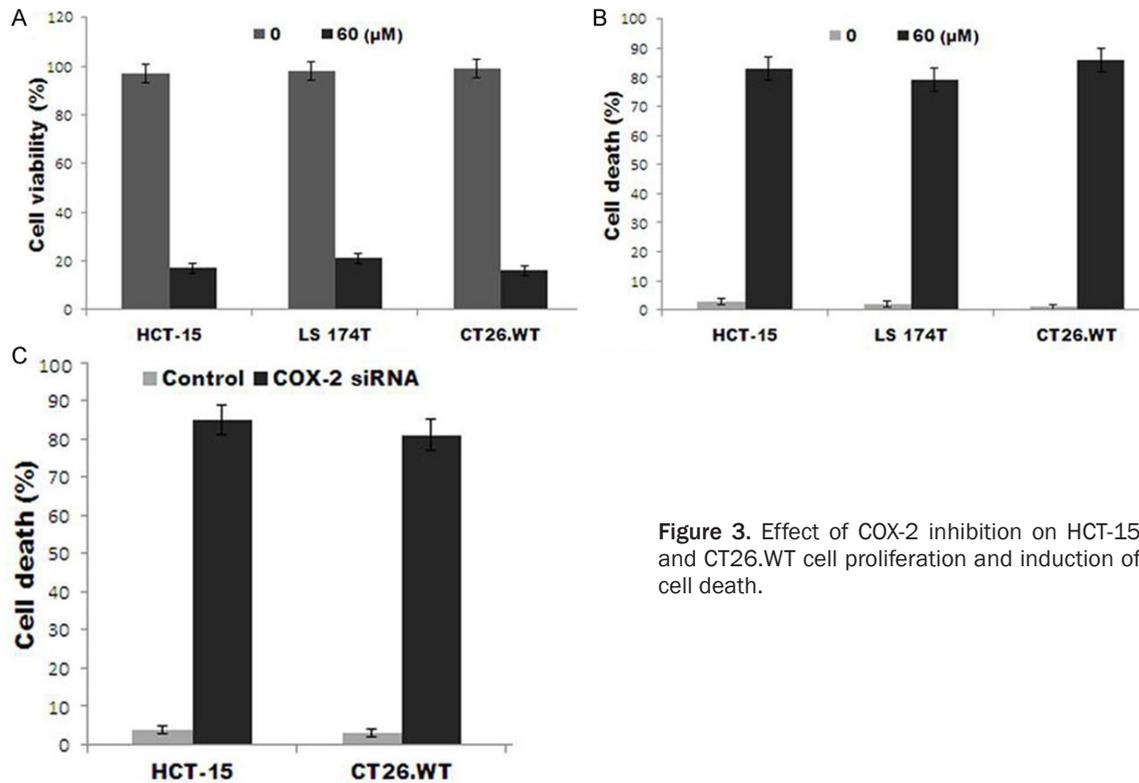


Figure 3. Effect of COX-2 inhibition on HCT-15 and CT26.WT cell proliferation and induction of cell death.

174T and CT26.WT colon cancer cells on treatment with RAC. Treatment of these colon cancer cells with various concentrations of RAC (0, 5, 10, 15, or 20 $\mu\text{g}/\text{mL}$) for 72 h resulted in a significant reduction in cell viability. In HCT-15, LS 174T and CT26.WT cells the cell viability was reduced to 22, 20 and 19% respectively at 20 $\mu\text{g}/\text{mL}$ after 72 h (**Figure 1A, 1B**).

RAC induces apoptotic cell death of human colon cancer cells

We used Annexin V-conjugated Alexa Fluor 488 (Alexa488) Apoptotic Detection kit, to examine RAC-induced apoptosis in HCT-15 and CT26.WT colon cancer cells. RAC treatment of HCT-15 cells for 72 h resulted in a highly significant dose-dependent enhancement in the numbers of cells in the early and late stages of apoptosis (**Figure 1C**). The percentage of apoptotic cells increased from 32.4 ± 3 , 45.0 ± 3 to $72.6 \pm 5\%$ respectively at 10, 15 and 20 $\mu\text{g}/\text{mL}$ compared to 3.7% (control) at 0 $\mu\text{g}/\text{mL}$ (**Figure 1D**). Similarly in CT26.WT cells the apoptotic cell percentage increased from 28.6 ± 3 , 41.2 ± 3 to $65.4 \pm 5\%$ on treatment with 10, 15 and 20 $\mu\text{g}/\text{mL}$ concentrations of RAC after 36 h compared to $2.9 \pm 1\%$ in control (**Figure 1C**,

1D). Thus, suggesting that human colon cancer cells are sensitive to RAC-induced apoptosis.

Human colon cancer cells overexpress COX-2 and exhibit enhanced PGE2 production

Western blot analysis showed higher levels of COX-2 expression in the HCT-15, LS 174T and CT26.WT colon cancer cells compared to the normal CCD-18Co colon cell line (**Figure 2A**). The homogenates of the equal numbers of cells demonstrated that the levels of PGE2 were higher in the colon cancer cell lines than the normal human colon cells (**Figure 2B**). As had been observed for the levels of COX-2, the concentrations of PGE2 were higher in the HCT-15, LS 174T and CT26.WT cell lines than the normal cancer cell line tested.

RAC reduces the constitutive overexpression of COX-2 and PGE2 production in colon cancer cell lines

HCT-15, LS 174T and CT26.WT cells were treated with various concentrations of RAC (0, 5, 10, 15, or 20 $\mu\text{g}/\text{mL}$) for 72 h and the levels of COX-2 expression in the cell lysates were then determined using Western blot analysis. The

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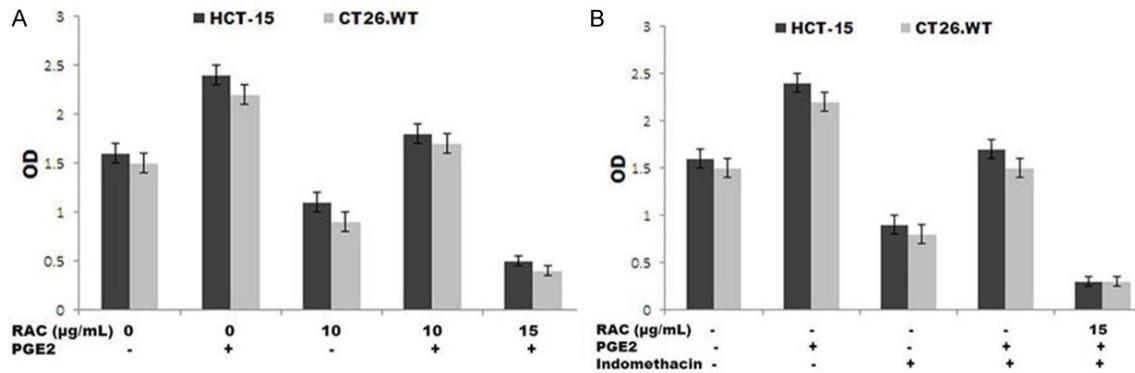


Figure 4. Effect of RAC on inhibition of PGE2-induced cell proliferation in colon cancer cells.

results revealed that the treatment of colon cancer cells with RAC resulted in a dose-dependent inhibition of COX-2 expression in the tested human colon cancer cell lines (**Figure 2C**). Since overexpression of COX-2 increases PG metabolites, the PGE2 expression was also determined. The results showed a dose-dependent inhibition of PGE2 production in HCT-15 and CT26.WT cell lines with RAC after 72 h compared with un-treated cells (**Figure 2D**).

Treatment of colon cancer cells with indomethacin, a pan-inhibitor of COX, inhibits cell growth and increases cell death

To confirm that the inhibitory effect of RAC on colon cancer cell growth/proliferation is mediated through inhibition of COX-2 expression, indomethacin, a COX inhibitor was used.

Treatment of HCT-15 and CT26.WT cells with 60 µmol/L indomethacin for 48 hours resulted in reduction of growth of the cells and cell viability compared with non-indomethacin-treated controls ($P < 0.01-0.001$). The results of the trypan blue exclusion assay revealed that the percentage of dead cells was significantly increased ($P < 0.05-0.001$) at 60 µmol/L doses of indomethacin (**Figure 3A, 3B**). Since COX-2 overexpression leads to cancer cell proliferation, indomethacin induced COX-2 inhibition causes induction of colon cancer cell death and inhibition of colon cancer cell proliferation.

Knockdown of COX-2 leads to the inhibition of cell growth and an the increase in cell death in colon cancer cells

We also investigated the effect of siRNA-mediated suppression of COX-2 in colon cancer

cells. HCT-15 and CT26.WT cells on transfection with siRNA showed a significant reduction in cell growth and induction of cell death (64-68%, $P < 0.001$) after 48 hours compared to control HCT-15 and CT26.WT cells (**Figure 3C**).

RAC Inhibit PGE2-Induced cell proliferation of colon cancer cells

Treatment of colon cancer cells with PGE2 resulted in a significant increase in cell proliferation after 48 h compared with PGE2 untreated cells (**Figure 4A**). However when HCT-15 and CT26.WT cells were pre-treated with 20 µg/mL concentration of RAC for 48 h PGE2-induced cell proliferation was inhibited (**Figure 4A**).

We also studied the effect of RAC on colon cancer cells treated with PGE2 (10 µmol/L) or indomethacin (40 µmol/L) or combination of the two. HCT-15 and CT26.WT cells treated with PGE2 showed increased proliferation compared to non-PGE2-treated cells. On the other hand, indomethacin treatment inhibited the proliferation in both PGE2-stimulated cells as well as PGE2-untreated cells. However, treatment with a combination of BAC + indomethacin decreased the PGE2-stimulated cellular proliferation of both HCT-15 and CT26.WT cells synergistically (**Figure 4B**).

RAC blocks the up-regulation of PGE2 receptors in colon cancer cells

Results from reverse transcription-PCR analysis of HCT-15 and CT26.WT cells treated with various concentrations of RAC (0, 5, 10, 15, or 20 µg/mL) for 48 h showed a dose-dependent decrease in the levels of EP1 and EP4 tran-

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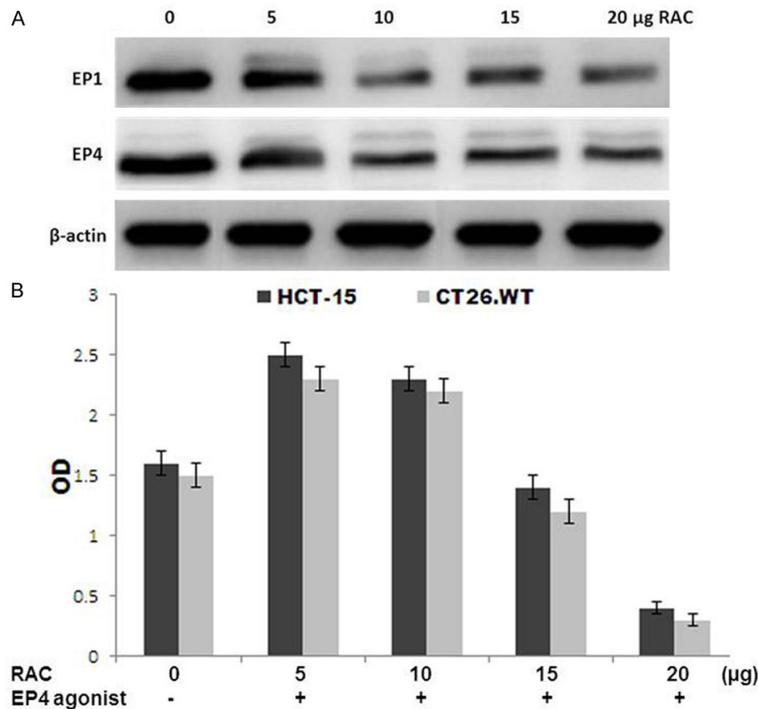


Figure 5. Effect of various concentrations RAC on inhibition of expression of PGE2 receptors EP1 and EP4 in HCT-15 and CT26.WT cells.

scripts (**Figure 5A**). The inhibitory effect of BAC on EP1 was less prominent than EP4 whereas the levels of EP2 or EP3 transcripts remained unchanged. Western blot analysis also revealed that levels of EP1 and EP4 were reduced in a dose-dependent manner on treatment with RAC. Treatment of HCT-15 and CT26.WT cells with the EP4 agonist resulted in the significant enhancement of cellular proliferation ($P < 0.01$) (**Figure 5B**). Treatment of cells with various concentrations of RAC significantly inhibited ($P < 0.01-0.001$) EP4 agonist-induced proliferation in a dose-dependent manner. These data suggest that the stimulation of PGE2 receptor in colon cancer cells has a role in cell proliferation, and that RAC inhibit the colon cancer cell proliferation, at least in part, by inhibiting the levels of PGE2 receptor.

Effect of dietary RAC on the levels of COX-2, PGE2, and PGE2 receptors on colon tumor xenografts in athymic nude mice

We investigated the effect of RAC in vivo on the levels of COX-2, PGE2, and the receptors of PGE2 in the tumor xenografts of HCT-15 and CT26.WT in mice. The results from Western blot analysis revealed that COX-2 levels were higher in tumor xenografts from mice fed the diet with-

out RAC compared to those grown with diet supplemented with RAC (**Figure 6B**). RAC supplemented diet also caused reduction of PGE2 levels in the tumor xenograft samples of HCT-15 (62%) and CT26.WT (50%) cells in mice. In addition RAC supplemented diet also lead to reduction in the levels of the PGE2 receptors EP1, EP3, and EP4 in the tumor xenografts from control mice (**Figure 6A**).

Discussion

It is reported that COX-2 overexpression is associated with tumor invasion, angiogenesis, and decrease of host immunity along with enhancement of resistance to apoptosis by tumor cells [24]. PGE2, a metabolic product of COX-2 mediates most of the protumorigenic effects and its high concentration creates feasible microenvironment for tumor growth. Therefore, discovery of a potent COX-2 inhibitor can be a promising therapeutic strategy for the treatment of colon cancer.

Retinoic acid is reported to be an effective agent in the treatment of epithelial and hematological malignancies such as breast cancer [25], head and neck cancer [26], ovarian adenocarcinoma [27], human malignant gliomas [28], and acute promyelocytic leukemia (APL) [29]. However, its side effects such as acute retinoid resistance, mucocutaneous dryness, headache, and cancer relapse after a brief remission [30] along with poor aqueous solubility [31, 32] hinder its clinical application. These drawbacks of RA have led to its chemical modification to develop the analogues free from such drawbacks.

The present study demonstrates that RAC inhibits colon cancer cell growth in vitro and in vivo. The results revealed that the treatment of colon cancer cells with RAC induces apoptotic cell death, and that is associated with the inhibition of COX-2 expression and PGE2 production. The colon cancer cells overexpress COX-2, and the inhibition of COX-2 by RAC may be

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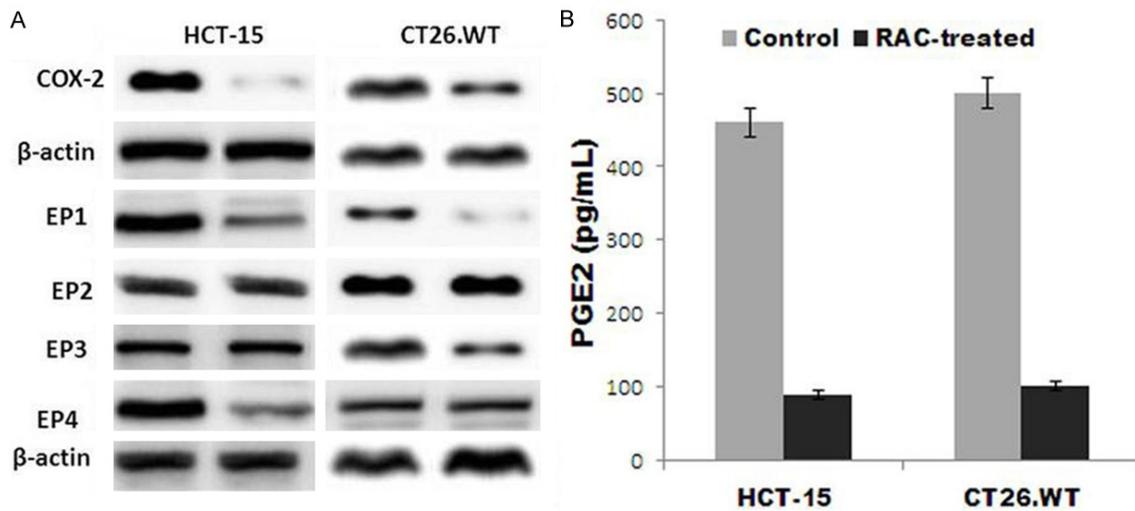


Figure 6. Effect of dietary RAC on inhibition of the growth of HCT-15 and CT26.WT colon cancer cells grown as xenografts in athymic nude mice.

responsible for the apoptosis of these cells. The strong evidence for this is provided by the fact that the treatment of the colon cancer cells with pan-COX inhibitor, indomethacin resulted in a significant decrease in cell proliferation and induction of apoptotic cell death. Similar effects were also observed when colon cancer cells, HCT-15 and CT26.WT were transfected with COX-2 siRNA. It has been reported that COX-2 inhibitors can induce apoptosis of colon cancer cells; however, although certain COX-2 inhibitors primarily induce apoptosis, others may predominantly induce growth arrest [33].

It is well known that PGE2 exerts its multiple actions through four G protein-coupled receptors, EP1, EP2, EP3, and EP4 [31] that can stimulate epithelial cell growth and invasion and promote cellular survival [34, 35]. We observed that HCT-15 and CT26.WT cells express the PGE2 receptors, EP1 and EP4, and that the expression of EP1 and EP4 was reduced when cells were treated with RAC in vitro. These data suggest that the inhibition of the EP1 and EP4 levels by RAC may contribute to the inhibition of tumor cell growth and induction of apoptosis of colon cancer cells. This assumption is based on the findings that PGE2 receptors coupled to the G α S, and ligand binding has been reported to increase cyclic AMP levels leading to the activation of PKA and Akt [36]. Akt and PKA activation can mediate prosurvival pathways through the inactivation of proapoptotic proteins [37, 38]. Our results are consistent with the report

that PGE2 protected gastric mucosal cells in vitro from ethanol-induced apoptosis via EP1 and EP4 activation [39]. The inhibitory effect of RAC on colon cancer cell proliferation through the inhibitory effect on EP1 or EP4 was further confirmed by treating the cells with EP4 agonist. We found that the treatment of HCT-15 and CT26.WT cells with EP4 agonist (PGE1 alcohol) resulted in enhanced cell proliferation, and that EP4 agonist-induced cell proliferation was inhibited by the treatment of cells with RAC. This observation further supports the concept that the inhibition of PGE2 receptors by RAC may have contributed to the inhibition of proliferation and induction of apoptosis in colon cancer cells.

To prove that RAC can inhibit the growth of colon cancer cells in vivo, the colon tumor xenografts grown in athymic mice were analyzed for the expression of COX-2, PGE2, and PGE2 receptors. The results revealed that the inhibition of tumor xenograft growth in athymic nude mice fed a diet supplemented with RAC was associated with the inhibition of COX-2 and PGE2 expression along with decrease in the levels of PGE2 receptors, EP1, EP3, and EP4. Thus, suggesting that the protective effects of RAC on the growth of colon cancer cells in vivo are induced through the inhibition of PGE2 and PGE2 receptors.

In conclusion, RAC is a potent chemotherapeutic agent for the inhibition of cell proliferation

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and induction of apoptosis in human colon cancer cells in vitro and in tumor xenograft growth in vivo. RAC leads to inhibition of COX-2 and PGE2 expression indicating the role of PGE2 receptors in this process.

Acknowledgements

This study was not financially supported by any research organization.

Disclosure of conflict of interest

None.

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References

- [1] Smith WL and Dewitt DL. Prostaglandin endoperoxide H synthases-1 and -2. *Adv Immunol* 1996; 62: 167-215.
- [2] Williams CS, Mann M and DuBois RN. The role of cyclooxygenases in inflammation, cancer, and development. *Oncogene* 1999; 18: 7908-7916.
- [3] Half E, Tang XM, Gwyn K, Sahin A, Wathen K and Sinicrope FA. Cyclooxygenase-2 expression in human breast cancers and adjacent ductal carcinoma *in situ*. *Cancer Res* 2002; 62: 1676-1681.
- [4] Kargman SL, O'Neill GP, Vickers PJ, Evans JF, Mancini JA and Jothy S. Expression of prostaglandin G/H synthase-1 and -2 protein in human colon cancer. *Cancer Res* 1995; 55: 2556-2559.
- [5] Molina MA, Sitja-Arnau M, Lemoine MG, Frazier ML and Sinicrope FA. Increased cyclooxygenase-2 expression in human pancreatic carcinomas and cell lines: growth inhibition by nonsteroidal anti-inflammatory drugs. *Cancer Res* 1999; 59: 4356-4362.
- [6] Ristimaki A, Sivula A and Lundin J. Prognostic significance of elevated cyclooxygenase-2 expression in breast cancer. *Cancer Res* 2002; 62: 632-635.
- [7] Sano H, Kawahito Y and Wilder RL. Expression of cyclooxygenase-1 and -2 in human colorectal cancer. *Cancer Res* 1995; 55: 3785-3789.
- [8] Sinicrope FA, Lemoine M, Xi L, Lynch PM, Cleary KR, Shen Y and Frazier ML. Reduced expression of cyclooxygenase 2 proteins in hereditary nonpolyposis colorectal cancers relative to sporadic cancers. *Gastroenterology* 1999; 117: 350-358.
- [9] Soslow RA, Dannenberg AJ, Rush D, Woerner BM, Khan KN, Masferrer J and Koki AT. COX-2 is expressed in human pulmonary, colonic, and mammary tumors. *Cancer* 2000; 89: 2637-2645.
- [10] Tucker ON, Dannenberg AJ and Yang EK. Cyclooxygenase-2 expression is up-regulated in human pancreatic cancer. *Cancer Res* 1999; 59: 987-990.
- [11] Van Rees BP, Saukkonen K, Ristimaki A, Polkowski W, Tytgat GN, Drillenburger P and Offerhaus GJ. Cyclooxygenase-2 expression during carcinogenesis in the human stomach. *J Pathol* 2002; 196: 171-179.
- [12] Wolff H, Saukkonen K, Anttila S, Karjalainen A, Vainio H and Ristimaki A. Expression of cyclooxygenase-2 in human lung carcinoma. *Cancer Res* 1998; 58: 4997-5001.
- [13] Yamagata R, Shimoyama T, Fukuda S, Yoshimura T, Tanaka M and Munakata A. Cyclooxygenase-2 expression is increased in early intestinal-type gastric cancer and gastric mucosa with intestinal metaplasia. *Eur J Gastroenterol Hepatol* 2002; 14: 359-363.
- [14] Takahashi M, Mutoh M, Kawamori T, Sugimura T and Wakabayashi K. Altered expression of beta-catenin, inducible nitric oxide synthase and cyclooxygenase-2 in azoxymethane-induced rat colon carcinogenesis. *Carcinogenesis* 2000; 21: 1319-1327.
- [15] Eberhart CE, Coffey RJ, Radhika A, Giardiello FM, Ferrenbach S and DuBois RN. Up-regulation of cyclooxygenase 2 gene expression in human colorectal adenomas and adenocarcinomas. *Gastroenterology* 1994; 107: 1183-1188.
- [16] Oshima M, Dinchuk JE and Kargman SL. Suppression of intestinal polyposis in Apc delta716 knockout mice by inhibition of cyclooxygenase 2 (COX-2). *Cell* 1996; 87: 803-809.
- [17] Cao Y, Pearman AT, Zimmerman GA, McIntyre TM and Prescott SM. Intracellular unesterified arachidonic acid signals apoptosis. *Proc Natl Acad Sci U S A* 2000; 97: 11280-11285.
- [18] Dempke W, Rie C, Grothey A and Schmol HJ. Cyclooxygenase-2: a novel target for cancer chemotherapy? *J Cancer Res Clin Oncol* 2001; 127: 411-417.
- [19] Rozic JG, Chakraborty C and Lala PK. Cyclooxygenase inhibitors retard murine mammary tumor progression by reducing tumor cell migration, invasiveness and angiogenesis. *Int J Cancer* 2001; 93: 497-506.
- [20] Sheng H, Shao J, Morrow JD, Beauchamp RD and DuBois RN. Modulation of apoptosis and Bcl-2 expression by prostaglandin E2 in human colon cancer cells. *Cancer Res* 1998; 58: 362-366.

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- [21] Backlund MG, Mann JR and Holla VR. 15-Hydroxyprostaglandin dehydrogenase is down-regulated in colorectal cancer. *J Biol Chem* 2005; 280: 3217-3223.
- [22] Yan M, Rerko RM and Platzer P. 15-Hydroxyprostaglandin dehydrogenase, a COX-2 oncogene antagonist, is a TGF-beta-induced suppressor of human gastrointestinal cancers. *Proc Natl Acad Sci U S A* 2004; 101: 17468-17473.
- [23] Tai HH, Ensor CM, Tong M, Zhou H and Yan F. Prostaglandin catabolizing enzymes. *Prostaglandins Other Lipid Mediat* 2002; 68-69: 483-493.
- [24] Riedl K, Krysan K and Pöld M. Multifaceted roles of cyclooxygenase-2 in lung cancer. *Drug Resist Updates* 2004; 7: 169-84.
- [25] Kalmekarian, GP, Jasti RK, Celano P, Nelkin BD and Marby M. All-trans retinoic acid alters myc gene expression and inhibits in vitro progression in small-cell lung cancer. *Cell Growth Differ* 1994; 5: 55-60.
- [26] Giannini F, Maestro R, Vukosa T, Vljevic T, Pomponi F and Boiocchi M. All trans, 13-cis and 9-cis retinoic acids induce a fully reversible growth inhibition in HNSCC cell lines: implications for in vivo retinoic acid use. *Int J Cancer* 1997; 70: 194-200.
- [27] Krupitza G, Hulla W, Harant H, Dittrich E, Kallay E and Huber H. Retinoic acid induced death of ovarian carcinoma cells correlates with c-myc stimulation. *Int J Cancer* 1995; 61: 649-659.
- [28] Defer GL, Adle-Biassette H, Ricolfi F, Martin L, Authier FJ, Chomienne C, Degos L and Degos JD. All-trans retinoic acid in relapsing malignant gliomas: clinical and radiological stabilization associated with the appearance of intratumoral calcifications. *J Neurooncol* 1997; 34: 169-177.
- [29] Huang EJ, Ye YC, Chen SR, Chai JR, Lu JX, Zhao L, Gu LJ and Wang ZY. Use of all-trans retinoic acid in the treatment of acute promyelocytic leukemia. *Blood* 1988; 72: 567-572.
- [30] Muindi JRF, Frankel SR, Miller WH, Jakubowski A, Scheinberg DA, Young CW, Dmitrovsky E and Warrell RP. Continuous treatment with all-trans-retinoic acid causes a progressive reduction in plasma drug concentrations: implications for relapse and retinoid "resistance" in patients with acute promyelocytic leukemia. *Blood* 1992; 79: 299-303.
- [31] Lehman PA, Slattery JT and Franz TJ. Percutaneous absorption of retinoids: influence of vehicle, light exposure, and dose. *J Invest Dermatol* 1988; 91: 56-61.
- [32] Szuts EZ and Harosi FI. Solubility of retinoids in water. *Arch Biochem Biophys* 1991; 287: 297-304.
- [33] Thun MJ, Henley SJ and Patrono C. Nonsteroidal anti-inflammatory drugs as anticancer agents: mechanistic, pharmacologic, and clinical issues. *J Natl Cancer Inst* 2002; 94: 252-66.
- [34] Tsujii M and DuBois RN. Alterations in cellular adhesion and apoptosis in epithelial cells overexpressing prostaglandin endoperoxide synthase *Cell* 1995; 83: 493-501.
- [35] Sheng H, Shao J, Washington MK and DuBois RN. Prostaglandin E2 increases growth and motility of colorectal carcinoma cells. *J Biol Chem* 2001; 276: 18075-81.
- [36] Regan JW. EP2 and EP4 prostanoid receptor signaling. *Life Sci* 2003; 74: 143-53.
- [37] Lizcano JM, Morrice N, Cohen P. Regulation of BAD by cAMP-dependent protein kinase is mediated via phosphorylation of a novel site, Ser155. *Biochem J* 2000; 349: 547-57.
- [38] Shimamura H, Terada Y, Okado T, Tanaka H, Inoshita S, Sasaki S. The PI3-kinase-Akt pathway promotes mesangial cell survival and inhibits apoptosis in vitro via NF- κ B and Bad. *J Am Soc Nephrol* 2003; 14: 1427-34.
- [39] Hoshino T, Tsutsumi S, Tomisato W, Hwang HJ, Tsuchiya T, Mizushima T. Prostaglandin E2 protects gastric mucosal cells from apoptosis via EP2 and EP4 receptor activation. *J Biol Chem* 2003; 278: 12752-8.
- [40] Reagan-Shaw S, Nihal M, Ahmad N. Dose translation from animal to human studies revisited. *FASEB J* 2007; 22: 659-61.