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In Vitro Evaluation of Apoptotic Induction of Butyric Acid Derivatives in Colorectal Carcinoma Cells

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Abstract. *Background/Aim: Butyric acid, a short chain fatty acid, plays an important role in the prevention of colon cancer. The aim of this study was to analyze the growth inhibitory and apoptotic effect of butyric acid derivatives in colorectal cancer cells. Materials and Methods: Human colorectal carcinoma HCT116 cells, were treated with the IC_{50} concentration of sodium butyrate, indole-3-butyric acid, tributyrin and 2-amino-n-butyric acid. Comet assay, caspase-3 assay and cell-cycle analysis were used to analyze apoptosis. Results: Tributyrin and indole-3-butyric acid showed the least IC_{50} values at 24 h incubation. Butyric acid derivatives significantly activated caspase-3 activity compared with the control. Additionally, indole-3-butyric acid and tributyrin caused G_0/G_1 and G_2/M phase arrest. Conclusion: Butyric acid derivatives effectively induced apoptosis in HCT116 cells.*

The microorganisms present in the human colon markedly influence the biology of the host through dietary fiber fermentation in the colonic lumen. Diet plays a major role in shaping the gut microbiota throughout life span (1-3). The major dietary fiber fermentation products are short-chain fatty acids, which are responsible for the beneficial effects of gut bacteria on colonic health (4). Any imbalance in the colonic microbiota will lead to digestive diseases. One of the life threatening diseases associated with imbalanced diet and microbiota is colon cancer (5). Colon cancer is the second leading cause of cancer death and the third in terms of incidence worldwide accounting for over 1.8 million new cases each year (6). Studies have suggested that high intake of dietary fiber reduces the risk of colorectal cancer (7, 8). The presence of dietary fiber fermentation product, butyric acid, in the colonic lumen is linked to decreased incidence of colorectal cancer *via* gut microbiota (9). Butyrate producing gut bacterial strains have histone deacetylase (HDAC) inhibitory action (10); the tumor-suppressive function of butyric acid is mainly due to this HDAC inhibitory action (11).

The antitumor effect of butyric acid is attributed mainly to growth inhibition and apoptosis in various cells (12, 13). Cell death is driven by caspases through direct stimulation of pro-apoptotic molecules and induction of tumor suppressor function (14). Some derivatives and prodrugs of butyric acid have been shown to induce apoptosis in a variety of tumor cells *in vitro* (15, 16). Besides the HDAC inhibitory action, butyrate also affects colon cells by acting as a ligand for GPR109A. It is a G-protein coupled receptor for nicotinate (niacin), which is silenced in colon cancer cell lines and its re-expression induces apoptosis in the presence of butyrate or nicotinate (17). The short half-life of butyric acid restricts its therapeutic application in cancer cells (18). This study examined the basic mechanisms behind the anti-cancer activity of some butyric acid derivatives in colorectal cancer cell lines.

Materials and Methods

Cell culture. Human colorectal carcinoma cell line, HCT116, was obtained from the National Centre for Cell Sciences, Pune. Cells were cultured in RPMI1640 media (Sigma-Aldrich, Darmstadt, Germany) supplemented with 10% Fetal Bovine Serum (FBS) and antibiotic-antimycotic solution (1 ml/100 ml) in a humidified atmosphere at 37˚C with 5% CO_2 (19).

Determination of IC_{50} value of butyric acid derivatives in HCT116 cells. IC_{50} values of sodium butyrate (Himedia), indole-3-butyric acid (Fischer Scientific, Mumbai, India), tributyrin (Himedia), 2-amino-n-butyric acid (Sisco Research Laboratories Pvt. Ltd., Mumbai, India) and nicotinate (S D Fine-Chem Limited, Maharashtra, India) were determined using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay. Cells were treated with increasing concentrations (1-10 mM) of butyric acid derivatives, nicotinate (GPR109A receptor ligand) and 5-fluorouracil (Himedia) (Positive control) for 24, 48 and 72 h (15, 20). Stock solutions of indole-3-butyric acid and tributyrin were prepared in dimethyl sulfoxide (DMSO) and that of other compounds were prepared in phosphate buffered saline (PBS).

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Key Words: Butyric acid, colon cancer, GPR109A, HCT116, cell cycle, caspase-3.

Morphological assessment of apoptotic cells by Acridine Orange staining. To perform morphological analysis of cell nuclei, Acridine orange staining was performed. A total of 8.825×10^5 HCT 116 cells were seeded in cell culture flasks. Cells were treated with IC_{50} concentrations of butyric acid derivatives, nicotinate and 5-fluorouracil for 24 h. After 24 h of treatment, cells were harvested and resuspended in PBS. Then, the cells were stained with 5 μg/ml acridine orange in PBS and observed under a fluorescent microscope (Leica DM6 B) (21).

Comet assay. Comet assay was performed to analyse DNA damage. HCT116 cells were treated with IC_{50} concentration of butyric acid derivatives as well as Nicotinate for 24 h. A total of 100 μl of 0.5% normal melting agarose in PBS was dropped onto a microslide, covered with a coverslip, and then placed at 4˚C for 10 min. The coverslip was removed and 50 μl cell suspension was mixed with 50 μl of 1% low melting agarose and applied on top of the gel, coated over the microslide, covered with a coverslip, and placed at 4˚C for 10 min. The coverslip was again removed and a third coating with 50 μl of 0.5% low melting agarose was placed on the gel and allowed to set at 4˚C for 15 min. The coverslips were removed and the slides were immersed in an ice-cold lysis solution (2.5 M NaCl, 100 mM EDTA,10 mM Tris, 90 mM sodium sarcosinate, NaOH, pH 10, 1% Triton X-100 and 10% DMSO) and kept at 4˚C for 2 h. Then the slides were placed horizontally in an electrophoresis chamber filled with the buffer (300 mM NaOH, 1.2 mM EDTA) and the DNA was allowed to unwind for 30 min. Then, electrophoresis was carried out at 25 V and 300 mA for 20 min. The slides were removed and washed in a neutralization buffer (400 mM Tris, HCl, pH 7.5). Cells were stained with 20 μl of ethidium bromide (20 μg/ml) and the slides were scored for comets by fluorescence microscopy (Leica DM6 B) (22).

Caspase-3 assay. Caspase-3 activity was assessed using a colorimetric method. This assay is based on the detection of the amount of Ac-DEVD-p-NA substrate cleaved by cell lysates to release the colored p-NA (Para nitroanilline) molecule. HCT 116 cells (5000 cells/well) were treated with IC_{50} concentration of butyric acid derivatives as well as nicotinate for 24 h. Following treatment, the cells were washed in PBS and suspended in lysis buffer (50 Mm HEPES, PH 7.4, 5 mM Triton X-100, 5 mM DTT) for 15 min. Lysed cells were centrifuged at 16,000 rpm, at 4˚C for 15 min. Lysate protein concentrations were determined using the Bradford assay. For each assay, 10-60 μl of cell lysate were added to each tube containing 0.07 mM substrate in assay buffer (20 mM HEPES, PH 7.4, 0.1% Triton X100, 5 mM DTT, 2 mM EDTA), bringing the total volume of each well to 100 μl. Caspase-3 activity was assessed by measuring the optical density at 405 nm using an AM 2100 Microplate Reader (ALERE). Activity was expressed as μmol p-NA released per min per milligram of protein (23).

Cell cycle analysis. For analysing distribution of cells in the different phases of the cell cycle, HCT 116 cells (100000 cells/ml) treated with IC_{50} concentration of indole-3-butyric acid and tributyrin. Camptothecin (15 μM) was used as a standard control. The cells were fixed in cold 70% ethanol and stored at 4˚C. Cells were incubated in propidium iodide/RNase staining buffer and cell cycle was analysed using a BD FACS calibur (15). PI histogram of the gated cell singlets distinguished cells at the G_0/G_1, S, and G_2/M cycle phases. Gating of cell cycle phases was approximate and can be refined using software (Cell Quest Software, Version 6.0) analysis.

Table I. *The IC_{50} values of butyric acid derivatives at 24, 48 and 72 h.*

Test compounds	IC_{50} Values (mM)		
	24 h	48 h	72 h
Sodium butyrate	9.53±0.55	7.83±0.46	5.98±0.12
Tributyrin	4.94±0.19	5.01±0.38	3.30±0.22
Indole-3 butyric acid	6.28±0.10	5.84±0.16	4.39±0.25
2-Amino-n- butyric acid	8.09±0.21	5.75±0.02	4.65±0.10
Nicotinate	15.13±1.34	4.65±0.21	3.71±0.03
5-Fluorouracil	1.98±0.23	1.12±0.19	0.99±0.42

Statistical analysis. Data are presented as mean±standard deviation. One-way analysis of Variance (ANOVA) with Duncan's *post-hoc* test using SPSS 21 software, were used to assess the level of significance between means at 95% confidence interval ($p<0.05$).

Results

Determination of IC_{50} value of butyric acid derivatives. Cell viability assay was performed to evaluate the IC_{50} of butyric acid derivatives as well as nicotinate in HCT116 cells. Among the butyric acid derivatives, indole-3-butyric acid and tributyrin showed the lowest IC_{50} values at 24 h of incubation (6.28±0.10, 4.94±0.19 mM respectively). There was no significant difference between the IC_{50} values of indole-3-butyric acid and tributyrin at 48 and 72 h of incubation. The IC_{50} values of butyric acid derivatives as well as Nicotinate are shown in Table I.

Morphological assessment of apoptotic cells by Acridine Orange staining. Fluorescent microscopy was performed after staining cells treated with Butyric acid derivatives as well as nicotinate with acridine orange. The fluorescent images revealed that these compounds induced apoptosis of HCT116 cells (Figure 1). The detached population of cells treated with butyric acid derivatives showed the typical morphological characteristics of apoptosis such as condensed chromatin and fragmented nuclei compared with the untreated control cells.

Analysis of DNA damage by the comet assay. The DNA damage induced following treatment of cells with butyric acid derivatives was analysed using single-cell gel electrophoresis (comet assay) in agarose gel matrix. No comet like appearance was observed in untreated cells. After treatment of HCT116 cells with the IC_{50} concentration of butyric acid derivatives as well as nicotinate for 24 h, well-formed comets were observed (Figure 2).

Caspase-3 assay. Treatment of HCT116 cells with butyric acid derivatives, as well as nicotinate significantly induced caspase-3 activity compared with the untreated control cells (Figure

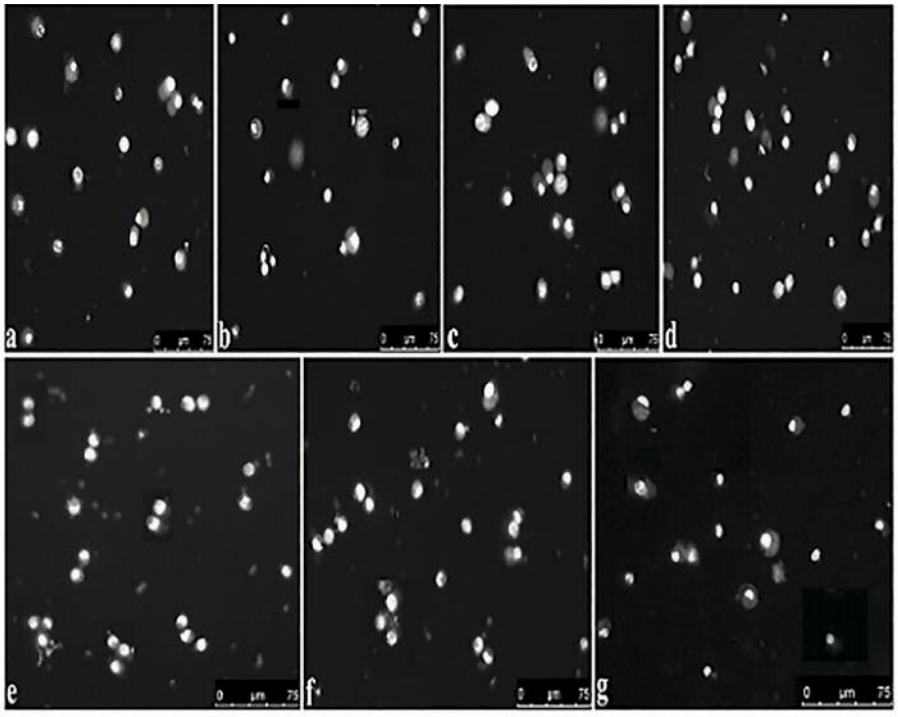

Figure 1. *HCT 116 cells stained with acridine orange after treatment with IC_{50} values of a) sodium butyrate, b) indole-3-butyric acid, c) tributyrin, d) 2-amino-n-butyric acid e) nicotinate, f) 5-fluorouracil, and g) untreated control.*

3). There was no significant difference between the caspase-3 activity in cells treated with butyric acid derivatives, nicotinate and 5-fluorouracil.

Effect of indole-3-butyric acid and tributyrin on the cell-cycle distribution of HCT116 cells. To analyse the mechanism of the the suppressive effect of indole-3-butyric acid and tributyrin on HCT116 cells, the changes in the cell cycle distribution were monitored by flow cytometry. The DNA content histogram is shown in Figure 4. Compared to the untreated control cells, 58.14±0.16% and 51.15±1.02% of HCT116 cells treated with indole-3-butyric acid and tributyrin arrested at the G_0/G_1 phase, respectively. Also, 20.91±0.86% and 17.07±0.06% of HCT116 cells treated with indole-3-butyric acid and tributyrin, respectively, arrested at the G_2/M phase compared to cells treated with camptothecin.

Discussion

In this study, we investigated the mechanisms through which butyric acid derivatives trigger apoptosis in HCT116 cells. The anti-proliferative and apoptotic activity of butyric acid, is mainly due to their histone hyperacetylation (24). Besides the butyric acid derivatives, the effect of nicotinate on HCT116 cells was also tested, because it acts as a ligand for GPR109A receptor protein. Studies have shown that the receptor is highly expressed in normal human colon tissue, but is silenced in colon carcinoma cells (25). Nicotinate and butyrate, suppress colon cancer cells in a GPR109A-dependent manner (26). Compared with other derivatives, the IC_{50} value of nicotinate was highest at 24 h. This result suggests that, as ligands, butyric acid derivatives are more cytotoxic in cancer cells than nicotinate. An earlier study has demonstrated induction of cytotoxicity by tributyrin emulsion in melanoma, with an IC_{50} of ~2 mM (27).

To investigate the involvement of apoptosis in butyric acid treated cells, morphological analysis using acridine orange staining was performed. The cellular changes of apoptosis include, cell shrinkage, masses of condensed chromatin and membrane blebbing (28). Cells treated with IC_{50} concentration of butyric acid derivatives as well as nicotinate showed early apoptosis features including membrane blebbing and chromatin condensation and these morphological features of apoptosis were comparable with those of cells treated with 5-Fluorouracil, the positive control. DNA damage is another hallmark of apoptosis. Well-formed comets were observed in the cells treated with indole-3-butyric acid, tributyrin and nicotinate. No comet like structures were found in the untreated control.

It was observed that apoptosis occurred in HCT116 cells treated with butyric acid derivatives *via* caspase-3 mediated signalling. The activity of caspase-3 in cells treated with

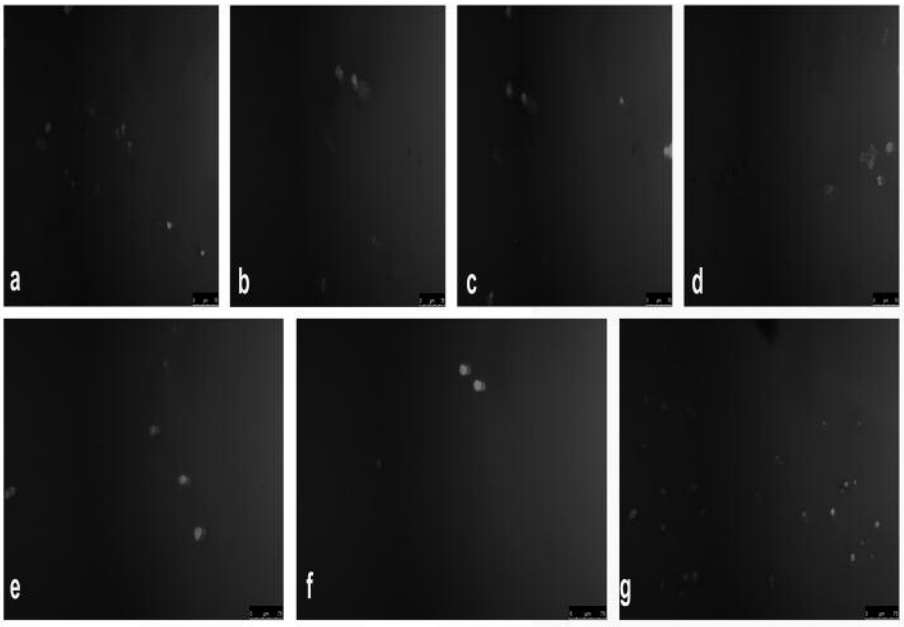

Figure 2. *Comet assay of HCT 116 cells treated with IC_{50} concentrations of a) sodium butyrate, b) indole-3-butyric acid, c) tributyrin, d) 2-amino-n-butyric acid, e) 5-fluorouracil and f) untreated control.*

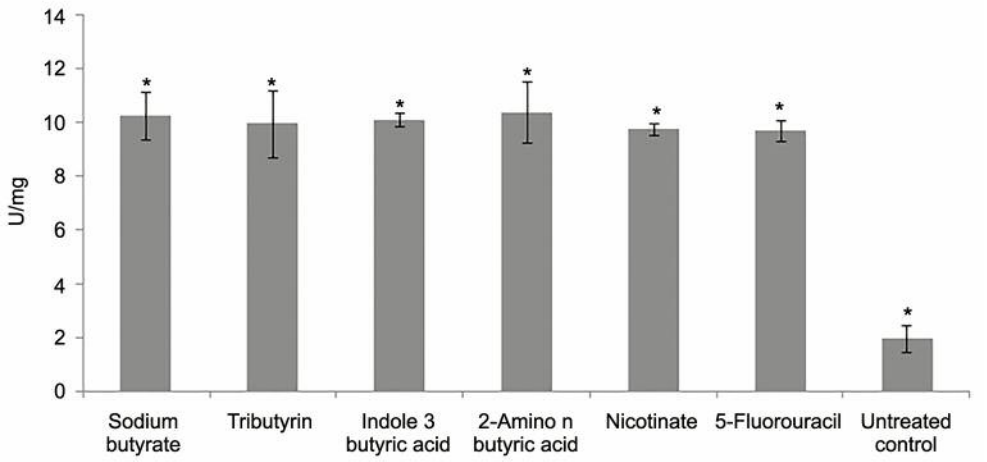

Figure 3. *Activity of caspase-3 in HCT 116 cells after treating with IC_{50} concentration of butyric acid derivatives, nicotinate and 5-fluorouracil at 24 h. Data are presented as mean±standard deviation in 6 wells per experiment from 3 independent experiments. *p<0.05 considered statistically significant.*

sodium butyrate, indole-3-butyric acid, tributyrin, 2-amino-n-butyric acid, nicotinate and 5-fluorrouracil, in U/mg of protein, were 10.28±0.9, 10.13±0.23, 9.98±1.25, 10.4±1.13, 9.76±0.22 and 9.71±0.4 respectively. The untreated control showed an activity of 1.98±0.49 U/mg of protein. Caspase-3 is a frequently activated protease in mammalian cell apoptosis (29). Its activation in colon cancer cells depends on peroxisome proliferator-activated receptor (30). Previous studies have suggested that butyrate induces apoptosis by caspase-3 activation in various kinds of colon cancer cell lines and is mediated *via* induction of caspase-3 mediated cleavage of poly ADP ribose polymerase (PARP) (31, 32).

Cell cycle analysis was performed to determine the effect of butyric acid derivatives on cell cycle progression of HCT116 cells. The derivatives, indole-3-butyric acid and tributyrin showed the least IC_{50} value at 24 h of incubation. So, cell cycle distribution following treatment with these two derivatives was analysed by flow cytometry. The results

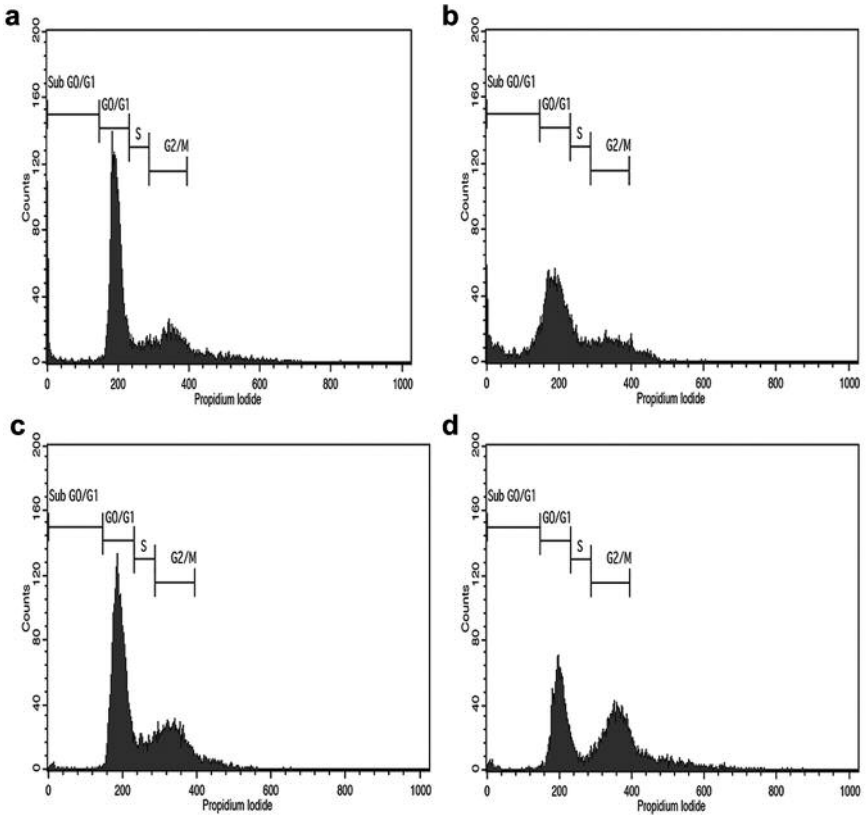

Figure 4. *DNA histogram of HCT116 cells treated with IC_{50} concentration of a) Indole-3-butyric acid, b) Tributyrin, c) Untreated control, d) Standard drug camptothecin.*

suggested that a higher number of cells were arrested at G_0/G_1 phase. A previous study has shown that butyrate causes G_1 arrest in cancer cell lines (33). Several studies have shown the cytotoxic effect of butyric acid in different cancer cells (12, 34) and it has been suggested that it induces apoptosis in colon cancer cells (35, 36). Our results clearly show that butyric acid derivatives are the most effective inducers of apoptosis in HCT116 cells.

Conclusion

The present study showed that butyric acid derivatives as well as nicotinate mediate caspase-3 dependent apoptotic pathway in HCT116 cells. The growth inhibitory effects are associated with alterations in cell cycle. Thus the present study suggested that butyric acid derivatives could be used to develop therapeutic interventions of colon cancer treatment.

Conflicts of Interest

The Authors have no conflicts of interest to declare regarding this study.

Authors' Contributions

LP contributed to the experimental work; all Authors participated in data analysis; HTBS coordinated the study. All Authors gave final approval for publication.

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