The anti-proliferative activity of Butyric acid derivatives in colon cancer cell lines

Thesis Submitted to
the University of Calicut in partial fulfillment of
the requirement for the award of the degree of

Doctor of Philosophy in Biochemistry

Submitted by

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Under the Guidance of

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CERTIFICATE

This is to certify that the Thesis entitled "**The anti-proliferative activity of Butyric acid derivatives in colon cancer cell lines**" submitted to University of Calicut, as partial fulfillment of the requirement for the award of the degree of Doctor of Philosophy in Biochemistry by **Liji P.**, embodies the results of bonafide research work carried out by her under my guidance and supervision at the Department of Life Sciences, University of Calicut. This Thesis has not previously formed the basis for the award of any degree, diploma, associateship, fellowship or other similar titles or recognition. The candidate has passed the course work of the Ph.D. programme in accordance with the UGC regulations. All corrections, as suggested by the examiners have been incorporated into this Thesis.

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DECLARATION

I hereby declare that the work presented in the Thesis entitled **"The anti-proliferative activity of Butyric acid derivatives in colon cancer cell lines"** submitted to the University of Calicut, as partial fulfillment of the requirement for the award of the degree of Doctor of Philosophy in Biochemistry, is original and carried out by me under the supervision of Dr. B.S.Harikumaran Thampi, Department of Life Sciences, University of Calicut. This has not been submitted earlier either in part or full for any degree or diploma of any university. All corrections, as suggested by the examiners have been incorporated into this Thesis.

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ABBREVIATIONS

2ANB	:	2-Amino-n-butyric acid
ANOVA	:	Analysis of Varience
BLAST	:	Basic Local Alignment Search Tool
C	:	Celcius
cm	:	centimetre
CRC	:	Colorectal cancer
DMSO	:	Dimethyl Sulfoxide
DNA	:	Deoxyribonucleic acid
DTT	:	Dithiothreitol
EDTA	:	Ethylene diamine tetra acetic acid
FACS	:	Fluorescence activated cell sorting
FBS	:	Fetal bovine serum
Hcl	:	Hydrochloric acid
HCT116	:	Human colorectal carcinoma cell line
HEPES	:	(4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
hr	:	hour
IBD	:	Inflammatory bowel diseases
I_3B	:	Indole-3-butyric acid
Kcl	:	Potassium chloride
KH_2PO_4	:	Potassium dihydrogen phosphate
mA	:	milliampere
ml	:	millilitre
mM	:	Millimolar
MTT	:	3-(4, 5-dimethyl thiazol-2-yl)-2, 5-diphenyl tetrazolium bromide

NaB	:	Sodium butyrate
Nacl	:	Sodium chloride
Na_2HPO_4	:	disodium hydrogen phosphate
NaOH	:	Sodium hydroxide
nm	:	nanometer
PBS	:	Phosphate buffered saline
PDB	:	Protein data bank
PI	:	Propidium iodide
p-NA	:	Para nitro anilline
RPMI	:	Roswell Park Memorial Institute Medium
RMSD	:	Root mean square deviation
rpm	:	Revelutions per minute
TB	:	Tributyrin
V	:	Volt
v/v	:	volume/volume
µM	:	micromolar
µg	:	microgram
g	:	gram

Chapter 1

INTRODUCTION

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1.1. Cancer: An overview

Cancer is a worldwide health problem and it becomes a big threat to human beings globally. According to International Agency for Research on Cancer, using the GLOBOCAN estimate, 18.1 million new cases and 9.6 million cancer deaths occur worldwide in 2018. Lung cancer and female breast cancer are the most commonly diagnosed cancer incidence (11.6%) followed by colorectal cancer (10.2%) and prostate cancer (7.1%) and for mortality, colorectal cancer (9.2%) , stomach cancer (8.2%) and liver cancer (8.2%) for both sexes combined worldwide (1). The major risk factors for death from cancer are mainly smoking, alcohol use, low fruit and vegetable intake, overweight and obesity (2). One of the life style dependent cancer is colorectal cancer and studies suggest that 47% of colorectal cancers could be prevented by appropriate lifestyle (3).

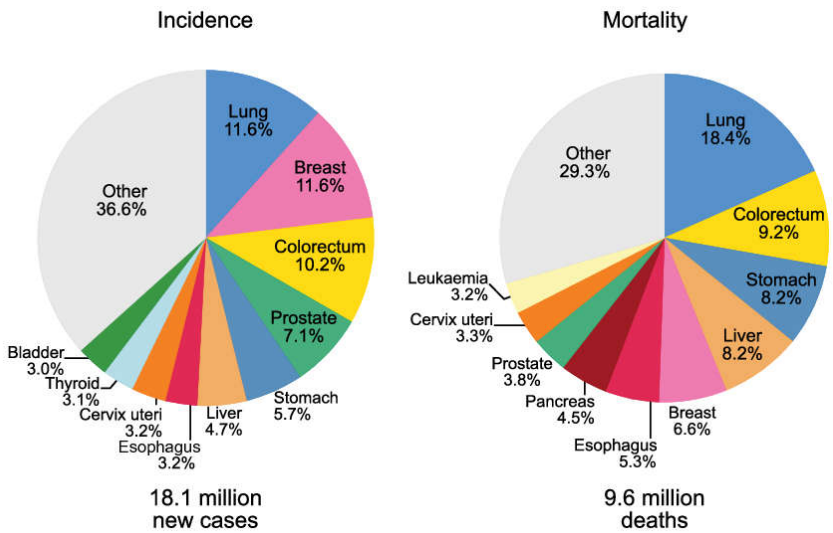


Figure 1.1: Pie diagram showing the incidence and mortality rate of various kinds of cancer in worldwide by GLOBOCAN estimation 2018 (1)

1.2. Colorectal Cancer

Colorectal cancer ranks the third in terms of incidence and second in terms of mortality, account for over 1.8 million new cases and 8, 81,000 deaths are estimated to occur in 2018 world wide (1). It usually begins as a polyp, a non cancerous growth that develops from the inner lining of colon and rectum. Adenocarcinoma, cancer arise from the inner lining of the colorectum, accounts approximately 96% of all colorectal cancers (4). The incidence and mortality rate of colorectal cancer vary widely worldwide and are rapidly rising in middle income and low income countries, increase in incidence with decrease in mortality seen in the countries with very high human development index (HDI) and the decrease in both incidence and mortality rate observed in countries with highest HDI. The rises in incidence rate are mainly due to the dietary and lifestyle factors. The best practices in cancer treatment and management in developed countries results the decline in mortality rate (5). Colorectal cancer is a heterogeneous disease and has three major forms, hereditary, sporadic and colitis associated colorectal cancer.

Heredetary Colorectal Cancer: Hereditary colorectal cancer accounts about 30 % of all cases and about 5 % cases are associated with the inherited mutations in cancer pre disposing genes (6). Genetic changes have a specific impact on tumor morphology and progression (7). Development of colorectal carcinoma and adenoma associated with tumour suppressor gene mutation (8). APC and K-*ras* mutation occur in ACF (Aberrent crypt foci), the precursor of colorectal cancer (9). The familial colorectal cancer was first described by Lynch in 1966 (Lynch et al. 1966). The hereditary colorectal cancer are categorized into polyposis and nonpolyposis syndrome. Colorectal cancer without

multiple colonic polyps called hereditary nonpolyposis colorectal cancer (HNPCC), accounts 3-6% of all colorectal cancers (11). It is mainly caused by the mutation in MMR (DNA Mismatch repair) gene Familial adenomatous polyposis (FAP) is caused by endodermal mutation of APC (Adenomatous polyposis coli) gene (12).

Sporadic Colorectal Cancer: Sporadic colorectal cancer is the major type, accounts approximately 75% of colorectal cancer (13). Sporadic colorectal cancer are of two types, hypermutated (16%) and nonhypermutated (84%). Hypermutated type is mainly due to the presence of microsatellite instability (MSI) as a result of mutation in mismatch repair genes and non hypermutated type is caused by the mutation including APC, K-Ras and p53 gene mutation (14).

Colitis associated colorectal cancer: It is mainly due to the complication of irritable bowel syndrome and is accounts almost 25 % of all colorectal cancer types. Chronic intestinal inflammation leads DNA damage which results in tumor development and progression (15).

1.3. Incidence of colorectal cancer

A wide geographical variation occurs in the incidence of colorectal cancer across the world. The highest incidence rate was observed in Europe, Australia, Northern America and Eastern Asia and it tend to be lower in parts of Africa and Southern Asia. The disease is considered as a marker of socio-economic development and the incidence rate directly related to the HDI (1). In India, cancer is the second most commonly diagnosed disease. According to Indian population census data, mortality rate is highest in India, it accounts

about 0.3 million deaths in each year. There is a sharp increase in the colorectal cancer incidence was seen in the group of above 45 years of age. In India almost 70 % of colorectal cancer incidence are due to the imbalanced diet (16). It is the fourth most common cause of cancer in males and third most common cause of cancer in females in India. The incidence of colorectal cancer in India is lower than those seen in western countries, but are rising with lifestyle and dietary factors (17). The annual incidence of colon cancer in India is 4.4 per 100000 in males and 3.9 per 100000 in females (18). The lower rate of incidence of colon cancer in India is believed to be due to the high intake of dietary fiber and low intake of meat. But the incidence rates are rising with change in life style (19). In kerala, 10-20% of all cancers are mainly due to dietary factors. It is approximately estimated that 35,000 of new cases occur in each year. About 30 % increase in the colorectal cancer are observed in Trivandrum cancer registry (20).

1.4. Molecular pathways of colorectal cancer

Colorectal cancer is a heterogeneous disease involving different molecular pathways. Genetic as well as epigenetic alterations occur in cell signalling pathways involving metabolism, proliferation, differentiation and apoptosis. The major molecular mechanism involved in colorectal cancer is chromosomal instability (CIN), microsatellite instability (MSI) and CPG island methylation (CIMP). 80-85% of colorectal cancer is included in CIN pathway. This type of instability is mainly affects the genes involved in cell function like Kras, adenomatous polyposis coli (APC) and phosphoinositide 3-kinase (PI3K). Mutation in APC gene causes the translocation of catenin to nucleus and leads to the transcription of tumorigenic genes. K-ras and PI3K mutation causes the MAPK (mitogen activated protein

kinase) activation. MSI pathway is associated with the alteration in DNA repair mechanism and leads the tumour suppressor gene inactivation. CPG island mutation causes hypermethylation of oncogenic promoters (21).

1.4.1. Wnt pathway

This pathway supports the intestinal epithelial renewal. APC is a tumour suppressor gene belongs to the Wnt pathway. The inactivation of APC gene is leads to the increased activation of Wnt signalling pathway which results in the accumulation of β-catenin. The accumulation of β-catenin in the cytoplasm leads its translocation in to the nucleus and it binds to T-cell factor (TCF) and increased proliferation occur (22, 23).

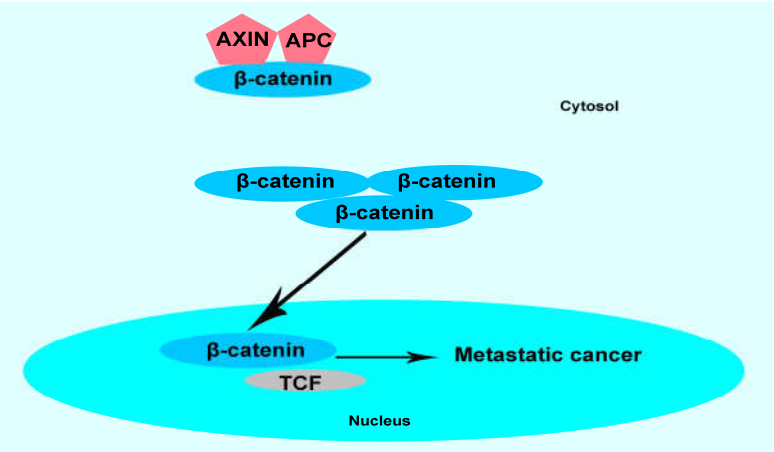


Figure 1.2: Figure illustrates the Wnt pathway through which cancer progresses. Mutation in APC gene is frequently observed in colorectal cancer.

1.4.2. RAS Pathway

The proto oncogene K-ras is involved in the conversion of adenoma to carcinoma. K-ras gene is mediates the extra cellular signal

transduction pathway and the mutation in the K-ras gene leads its permanent activation and enhances cell growth and proliferation. This pathway involved in initiation of carcinogenesis also (22).

p53 gene: The loss of function of p53 gene is occur in later stage of colorectal cancer. Mutation in this gene is causes the loss of cell cycle control and leads high cell proliferation rate. WAF-1 (Wild type p53 activated fragment) is a gene that suppresses the cancerous cell growth in p53 pathway. But the mutation in p53 gene down regulate the expression of this gene (22).

1.4.3. CIN pathway

Chromasomal instability (CIN) is the most common type of pathway in colorectal cancer. This pathway is mainly characterised by the alteration in region of chromosome and leads to DNA damage. The most common mutation is occur in APC and k-ras genes (23).

1.4.4. MSI pathway

About 15% of sporadic colorectal cancer and 95% of hereditary non polyposis colorectal cancer (HNPCC) are caused by Microsatellite instability (MSI) pathway. Alteration in the DNA mismatch repair mechanism causes the MSI and leads the highest rate of mutation in colon mucosa cells. MSI pathway also leads the mutation in pro-apoptotic tumour suppressor gene BAX and which causes the escape of cells from intrinsic apoptotic pathway (22).

1.4.5. CIMP pathway

Colorectal cancer also progresses through CpG (5'-cytosine-phosphate-Guanine-3') island methylator phenotype. The hyper

methylation of CpG di-nucleotide sequence occur in the promoter region of genes present in cell cycle regulation and apoptosis which causes the loss of gene expression. The hyper methylation leads the mutation of K-ras or BRAF genes which leads the mutation of Tumour suppressor gene P53 (TP53) and leads the metastatic cancer. CIMP pathway is observed in approximately 20-30% of colorectal cancer (22).

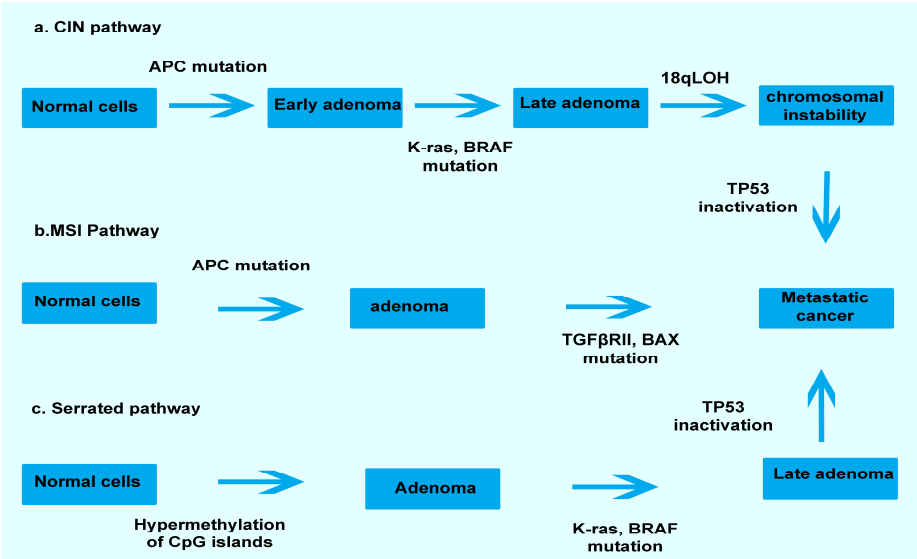


Figure 1.3: Figure illustrates the 3 major molecular pathways involved in colorectal cancer. **Figure 3a** is the chromosomal instability pathway in colorectal cancer. K-ras (Kirsten Rat Sarcoma viral oncogene homolog) and BRAF (Murine sarcoma viral oncogene) mutation leads the chromosomal instability. (8qLOH) Loss of heterogeneity of the long arm of chromosome 18q (contains SMAD4 and SMAD2 genes (Tumour suppressor genes mothers against decapertaplegic homolog 4 and 2) observed and resulted in inactivation of Tumour suppressor p53 gene (TP53) and metastatic cancer. **Figure 3b** shows the colorectal cancer pathway due to genetic instability which leads the mutation of TGFβRII (Transforming growth factor β receptor) and BAX (Apoptotic regulation gene Bcl-2 associated X) and metastatic cancer and **Figure 3c** shows the CpG island mutation (serrated pathway).

Other pathways

1.4.6. MicroRNA and inflammatory pathways

miRNA are short length non-coding RNA inhibits the mRNA transcription and regulate protein expression involved in cell differentiation and apoptosis. miRNA causes the early development of tumours. Inflammation leads to the initiation and progression of cancer. The enhanced level of Tumor necrosis factor- α enhances the level of tumor growth (22) .

1.5. Risk factors

1.5.1. Family history

Higher incidence of CRC was observed in people with positive family history compared with no family history. Approximately 20-25% of colorectal cancer incidences are depends on family history. The increased risk of colorectal cancer is observed in first degree relatives of patients with same disease. The prevalence of CRC with at-least one first degree relatives (FDR) was estimated between 3.1 and 10% and that of two FDR was between 0.3-0.34 %. The risk of CRC depends on the age of the person. Having an FDR with CRC diagnosis before age 50 years was uncommon, in older ages (above 65 years) which were about more than 3%. The risk was higher, in the incidence when the relative was diagnosed at younger age (24).

1.5.2. Inflammatory bowel disease

The patients with inflammatory bowel disease, ulcerative colitis and crohn's diseases are associated with increased risk of colorectal cancer. About 10-15% of colorectal cancer risks are associated with inflammatory bowel diseases (IBD) (25). Ulcerative colitis and Crohns disease are the two major inflammatory bowel diseases. Inflammation in the colon and rectum mucosa causes ulcerative colitis whereas inflammation in the any portion of digestive tract causes Crohn disease. The chance of developing colorectal cancer is estimated between 4 to 20 fold in patients with inflammatory bowel diseases (26). Oxidative stress in colitis also, leads to the development of cancer. During the oxidative stress condition, increased synthesis of Nitric oxide leads the formation of 8-Nitroguanine, an analogue of nucleobases , induces damage to the bases. Cytokines produced during the inflammatory action associated with the development of IBD associated colorectal cancer. Another key factor produced in inflammation process is Nuclear factor kappa B (NF-κB). Activation of NF-κB sometimes causes cancer. Tumour necrosis factor-alpha (TNF-α) is a factor associated with inflammation and which also cause DNA damage and leads malignant tumours (27).

1.5.3. Life style and dietary factors

Apart from the genetic factors, life style plays a major role in the progression of colorectal cancer (28). The major life style associated risk factors for colorectal cancers are obesity, alcohol, smoking and consumption of processed and red meat (29). Colorectal

cancer risk is directly associated with the alcohol consumption. Heavy alcohol drinkers had 60% more risk for developing cancer than non or light drinkers. Long term consumption of alcohol reduces the absorption vitamine B1, B2 and folic acid and the cells are more susceptible to oxidative stress and which leads to the cell proliferation and metastasis. Alcohol metabolised in our body by the enzymes, Alcohol dehydrogenase, catalase and cytochrome P450 to form acetaldehyde, it is a class 1 carcinogen and which leads to the damage in chromosome and also influence the metabolism of colonic microbes (30). Smoking, high red meat intake, diabetes and obesity were also associated with developing more than 20 % risk. Study suggests that increased physical activity is protective against colorectal cancer (31).

Many studies suggested that the use of tobacco increases the chance of getting colon cancer. Nitrosamine and polycyclic aromatic hydrocarbon are the major carcinogen present in tobacco. These compounds metabolised by cytochrome P450 and abnormal DNA forms and which leads to the gene mutation. Presence of nitrosamine leads to the production of reactive oxygen species and which induces the NF-kB mediated inflammatory pathway and cell proliferation occur (32).

In industrialised countries change in lifestyle and dietary factors are mainly responsible for colorectal cancer incidence. High intake of red and processed meat with low intake of dietary fiber is the major cause of cancer in western region of the world. Fruits, grains and vegetables are the major source of fiber. Dietary fiber has an ability to reduce the transit time through the intestinal tract. It also

helps for the dilution of contents in the colon and enhances the bacterial fermentation (32).

1.6. Treatment of colorectal cancer

Colorectal cancer treatment depends on tumour localisation stage and patient factors. Surgical resection involving the coloctomy is the major treatment option. Un-resectable tumours are treated by adjuvant chemotherapy (33). The major chemotherapeutic agents used for colorectal cancer are mentioned below.

Fluorouracil: Fluorouracil inhibits the synthesis of Thymidylate synthetase, an enzyme involved in the pyrimidine nucleotide synthesis.

Irinotecan: derivative of the natural alkaloid camptothecin which inhibits topoisomerase 1, enzyme that catalyses the breakage and joining of DNA strand during replication.

Oxaliplatin: It is a diaminocyclohexane platinum compound. It forms DNA adducts and inhibit replication and leads apoptosis.

Angiogenesis inhibitors: Angiogenesis process is mainly inhibited by suppressing the vascular endothelial growth factor (VEGF). VEGF stimulate the production of new blood vessels. Bevacizumab (Avastin) is the commonly used angiogenesis inhibitor.

Epidermal growth factor receptor inhibitor: Epidermal growth factor (EGFR) is a glycoprotein involved in the process of cell signalling and apoptosis. EGFR is expressed in the malignant tissues. Cetuximab and panitumumab are the epidermal growth factor receptor inhibitors used for the treatment of colorectal cancer (34).

1.7. Dietary fiber and colorectal Cancer

Dietary fiber plays an important role in the prevention of colorectal cancer. Diets with low fiber and high red meat increase the risk of colorectal cancer. Dietary fiber modulates the intestinal microbiota composition. Low fiber and high fat containing diet reduces the microbial taxa, especially the *firmicutes*, the butyrate producing bacteria reduces (35, 36). Many of the health benefits of dietary fiber are due to their fermentation into short chain fatty acids in the colon (37). Billions of bacteria resides in mammalian colon and they influence the biology of host including energy balance and immune functions (38, 39). The microorganisms present in human colon markedly influence the biology of the host through the dietary fiber fermentation in the colonic lumen. The probiotics were used as preventive agents in colorectal cancer in many clinical and pre-clinical studies are often linked to the production of short chain fatty acids in the gut (40). It plays an essential role in the maintenance of intestinal metabolism. The major probiotic group present in the digestive system are *Bifidobacterium* and the *Lactobacillus* genera (41). The prebiotic activity of fibers promote the beneficial bacteria in the gut and leads to the production of short chain fatty acids and could account the key mechanism for the protective effect of fibers on colon carcinogenesis (42).

1.7.1. Dietary fiber fermentation

Dietary fiber plays a major role for shaping the gut microbiota throughout the life time (43–45). Dietary fibers are the carbohydrate polymers with ten or more monomeric units which are neither digested nor absorbed in the human small intestine. Various types of dietary

fiber enhances and supports the diverse microbial composition in the gut. Fibers are mainly derived from cereals, nuts, vegetables, fruits, legumes and grains. The physiochemical characteristics like solubility and viscosity of fibers influences fermentation as well as the therapeutic effects. The carbohydrates which are not digested by the enzymes are the substrate for bacterial fermentation in the gastrointestinal tract. Soluble fibers like short chain fructooligosaccharide and pectins are metabolised in the gastrointestinal tract and that of less soluble fibers like cellulose partially fermented in the distal colon (46). The proximal part of colon is the principal site of fermentation where the substrate availability is high. The fermentation occurs in the anaerobic environment of the gut and the initial pathways are Glycolysis and Pentose phosphate pathway, which converts monosaccharide into Phosphoenolpyruvate (PEP). PEP is then converted into fermentation products, Acetate, Propionate and butyrate. The Bacteroidetes (gram negative), Firmicutes (gram-positive) and Actinobacteria (gram-positive) are the most abundant phyla present in the intestine. Acetate and propionate produced by bacteroidetes and the Firmicutes phylum produces butyrate as its primary metabolic product (47). Acetate, Propionate and butyrate produced in the colon at a molar ratio of approximately 3:1:1 respectively. The metabolism of acetate is lower in the colon due to its transportation to the liver. Acetate mediates lipogenesis in adipocytes and mammary glands. It also act as the major substrate for cholesterol synthesis. Propionate has a role in lipid lowering effect and it inhibits hepatic cholesterol synthesis. Butyrate is the energy source for colonocytes and almost 70 to 90% of butyrate is metabolised by

butyrate (48). Short-chain fatty acids are responsible for the beneficial effects of gut bacteria on colonic health. The major health benefit of short chain fatty acid in the colon is reduction in the luminal pH that helps for the enhancement of nutrient absorption as well as the inhibition of pathogenic microorganisms. Propionate mainly involved in gluconeogenesis pathway and that of acetate and butyrate participates in lipid biosynthesis (49).

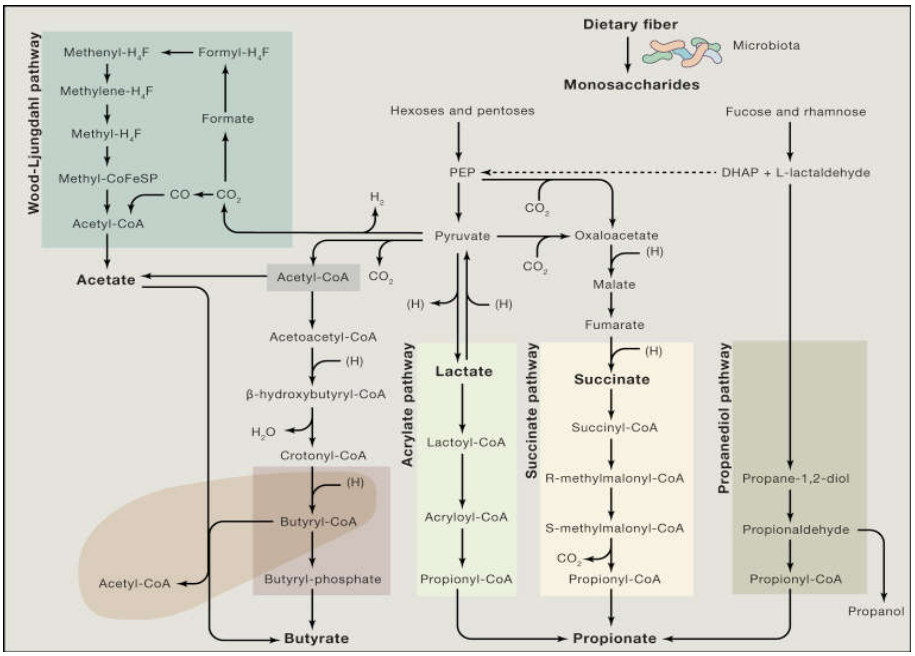


Figure 1.4: The diagram illustrates the metabolism of dietary fiber fermentation in the gastrointestinal tract and the production of short chain fatty acids (50)

1.8. Butyric acid

Butyric acid is a 4 carbon volatile short chain fatty acid essentially produced in the colon by dietary fiber fermentation. Butyrate is mainly involved in the maintenance of colonic health and is

the major energy source for colonocytes (51). The concentration of butyrate is maximum in caecum portion and reduces distally because of its absorption by mucosa and the decreased availability of fermentation substrates. The daily production of butyrate in the human large intestine is approximately >200 mM. The concentration of butyrate is 1000 fold higher in colonic lumen compared to portal vein (52). The absorption of butyric acid in the colon is by non ionic diffusion across the apical membrane of colonocytes (53). Proton coupled transportation was also proposed for the butyrate uptake into the luminal membrane vesicles (LMV) and this transportation was enhanced at P^H 5.5 (54). In addition to this transportation, a member of the sodium-coupled monocarboxylate transporters (SCMTs), solute carrier family 5 member 8 (SLC5A8) act as the primary transporter of butyrate (55).

Butyrate act as the ligand for G protein coupled receptors, GPR43 (Free fatty acid receptor 2 (FFAR2)) and GPR41 (Free fatty acid receptor 3 (FFAR3)) (56). GPR41 is present in adipose tissues and i mMune cells and GPR43 found in immune cells (57). In addition to this receptors, butyrate also act as the ligand for GPR109A, a G-protein coupled receptor for Nicotinate found in colonic epithelial cells (58). Many of the health benefits of butyric acid is due to its histone deacetylase inhibitory action. It determines the histone acetylation activity in chromatin structure and function. Thus butyrate plays an important role in prevention of cancer through these activities (59).

1.8.1. Anti-inflammatory action of butyrate

Butyrate is not only act as energy source for the colonocytes but also exerts anti-inflammatory effects. Butyrate prevents colonocytes from autophagy through its histone deacetylase inhibitory action (60). Interleukin-8 (IL-8) is a frequently elevated cytokine in inflammatory bowel disease. The administration of butyrate reduces the IL-8 concentration in the colonic crypt cells (61). Sodium butyrate suppresses the inflammation and maintains epithelium barrier integrity in colitis model by inhibiting the macrophages by the activation of GPR109A. Thus GPR109A can act as a valid therapeutic target in inflammatory bowel disease (62). Crohn's disease is an inflammatory bowel disease, which involves the pro inflammatory cytokines in its pathogenesis. Butyrate decreases the expression of pro inflammatory cytokines via the inhibition of nuclear factor kappa B (NFkB) activation. Activation of NFkB involved in Crohn's disease (63). The anti-inflammatory effect of butyrate is mediated by GPR43 in chondrocytes. Butyrate reduced the pro inflammatory mediators and inflammatory adipokines by inhibiting the major inflammatory signalling pathways like NF-kB, ERK/MAPKinase and AMPK (64).

1.8.2. Butyrate and Obesity

The studies suggest that the dietary supplementation of butyrate induces lower body fat percentage and reduces the adiposity (65). Oral administration of sodium butyrate can prevent diet induced obesity through the activation of the expression of adiponectin receptors and diminishes the expression of histone deacetylase- 1 (66). Butyrate

supplementation prevents the diet induced obesity, hyperinsulinaemia and hyper triglyceridaemia by promoting fat oxidation and brown adipose tissue activation (67). The diet induced body weight and adiposity prevented by sodium butyrate without altering the food intake or energy expenditure. The insulin tolerance test as well as the decreased respiratory exchange ratio also provides an evidence for the improved insulin sensitivity by sodium butyrate (68).

1.8.3. Effect of butyrate on Liver, Skeletal muscle and Adipose tissue

Butyrate exerts beneficial effects on peripheral tissues like liver, skeletal muscles and adipose tissues. There are several studies were reported about the efficiency of butyrate as well as its derivatives on various diseases. It has a protective role in insulin resistance and fatty liver. In the case of diet induced obesity, hepatic mitochondria were identified as the main target of butyrate and its derivatives in insulin sensitivity reaction. Butyrate improves the fatty acid oxidation and thereby leads the reduction of intracellular fat accumulation and oxidative stress (69). The oral supplementation of sodium butyrate protects against Non alcoholic fatty liver diseases (NAFLD) with an increased melatonin synthesis (70).

Skeletal muscle is the largest insulin responsive tissue and which utilizes blood glucose during metabolism. Butyrate reduces the obesity as well as the insulin resistance by acting on skeletal muscles. The short-term oral administration of sodium butyrate enhances the insulin sensitivity through the activation of adiponectin mediated

pathway as well as the stimulation of mitochondrial function in the skeletal muscles (66). Supplementation of butyrate during the gestation and lactation period influences the mitochondrial biogenesis of off springs through the GPR43 and GPR41 dependent pathway. The expression of these receptors enhances in the skeletal muscles of off springs (71).

Butyrate enhances the fat oxidation and promotes brown adipose tissue (67). In adipose tissues, butyrate has a capacity for fat storage and mobilisation as well as glucose uptake. So they enhances the energy metabolism by reducing the circulating free fatty acids and contribute a healthier adipocytes (72). Studies shows that the butyrate treatment increases the rate of lipolysis in adipocytes approximately 2-3 fold time. The mechanism behind this activity is histone deacetylase inhibition (73).

1.8.4. Role of butyrate in colon cancer prevention

Colorectal cancer is a diet dependent cancer and any alteration in metabolic pathway leads the tumor development and progression. Butyrate acts as the major energy source for colon. But in the colon cancer cells, glucose is the major source of energy. Butyrate reduces its own oxidation in cancerous colon cells by lowering the expression of short chain acyl-CoA dehydrogenase, an enzyme that catalyses the short chain fatty acid oxidation. The reduced activity of this enzyme is mainly due to the histone deacetylase inhibitory action of butyrate (74). Butyrate acts as the key mediator of protective effect of dietary fiber on colorectal carcinogenesis. Sodium butyrate inhibits the

growth of colon cancer cells by up-regulating the cell cycle inhibitor p21. The induction of p21 and the growth arrest occur through a mechanism involving the histone deacetylase inhibitory action (75). The class 2 histone deacetylases, HDAC4 act as the regulator of proliferation of colon cancer cells and they promote the growth of colon cancer cells invitro (76). Butyrate modulate histone acetylation in human HT29 colon cancer cells at physiological concentration and leads the silencing of genes responsible for cancer progression (Kiefer et al. 2006). Butyric acid elicits its anti-cancer activity by altering cellular metabolism. The treatment with butyrate decreases the ability of cancer cells to oxidise glucose. The cancer cells which are highly glycolytic can switch into butyrate utilizing phenotype to produce acetyl CoA. It also stimulate glutamine utilization and leads the inactivation of pyruvate dehydrogenase complex by hyperacetylating the histones at PDK4 gene promoter and upregulate the pyruvate dehydrogenase kinase (78).

Butyric acid plays a beneficial role in the management of colon cancer and display anti-cancer activity through the induction of apoptosis in various cancer cells (79, 80). Butyrate effectively inhibits the proliferation of cancerous colon cells than the non cancerous cells at physiologically relevant doses by regulating the p^{21} tumor suppressor protein expression (81). Butyrate is considered as a potent inducer of apoptosis in colorectal cancer cells. Butyrate induced apoptosis in colon cancer cells were mainly mediated through the mitochondrial pathway. The major step in this pathway is the alteration of bcl expression which leads the upregulation of bak

followed by the cytochrome-c translocation from mitochondria to the cytosol resulting the caspase cascade activation and apoptosis (82). The butyrate induced DNA fragmentation and upregulation of bak were preceded by the early stimulation of JNK (c-Jun N-terminal kinase) and AP1 (Transcription factor activation protein 1) (83).

One of the issue with the usage of butyric acid as an anti-cancer agent is its rapid metabolic clearance from the body. Butyric acid has short half-life, which considerably restricts its therapeutic application (84). Studies suggests that the rapid degradation and subsequent lack of efficacy of butyric acid is improved by some stable pro-drugs (85, 86). Tributyrin, a rapidly absorbed butyric acid prodrug inhibits the cancerous cells without affecting non cancerous cells. It is a natural prodrug found in milk fat and honey. Half life of tributyrin was approximately 40 min after oral administration (87).

Studies on tumour suppressive function of butyric acid has focused mostly on its action as a histone deacetylase (HDAC) inhibition. Besides this HDAC inhibitory action, butyric acid derivatives has exhibits anti-cancer activity in colon cancer cells by mediated as a ligand for GPR109A receptor. GPR109A is a G-protein coupled receptor for Nicotinate and mediates lipolytic effect (88). The receptor has an essential role in mediating the beneficial effects of gut microbiota and dietary fibers in colon and the anti-cancer activity of butyric acid is also related to GPR109A receptor (89). Gut bacteria plays an active role in the host intestinal tract and which promote the expression of

GPR109A which is obligatory for the bacterial fermentation product butyric acid (55). This receptor is silenced in colon cancer cells in humans and its re-expression induces apoptosis in the presence of butyrate (58).

1.8.4.1. GPR109A

The proteins GPR81, GPR109A and GPR109B are the G-Protein coupled receptors included in the group of Hydrocarboxylic acid (HCA) receptors and they share significant sequence homology. Hydrocarboxylic acids are the ligands of these receptors. Lactate is the ligand of GPR81 (HCA_1). Nicotinic acid as well as the beta-hydroxy butyrate acts as the ligand of GPR109A (HCA_2). This receptor is mostly found in mammalian species and they induces G-protein mediated anti-lipolytic effect in adipocytes. GPR109A receptor is also expressed in macrophages, dendritic cells and neutrophils. Apart from adipocytes and immune cells, the receptor is also expressed in intestinal epithelial cells (90). The lipid lowering effect of nicotinate is mainly depends on GPR109A proein (PUMA-G in mouse and HM74 in human). This receptor is highly expressed in adipose tissue. Binding of nicotinic acid with this receptor leads to the reduction in cAMP and shows anti-lipolytic and lipid lowering effect (91)

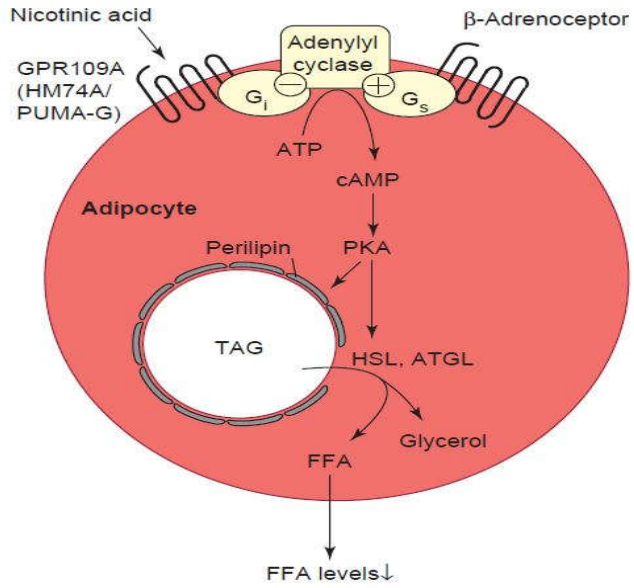


Figure 1.5: Figure illustrates the mechanism of action of GPR109A receptor in reducing free fatty acid level. The activation of GPR109A results in decreased cAMP and leads reduced free fatty acid level (92)

1.8.5. Alteration of mitochondrial apoptotic pathway by Butyrate

Butyrate plays an important role in the mitochondrial apoptotic pathway. Butyrate suppresses the Bcl-2 anti-apoptotic protein and enhances the pro-apoptotic protein Bax which leads to the release of cytochrome-c from mitochondria and oligomerisation of Apaf-1 (Apoptosis activating factor-1) occur. Caspase cascade activation and results in apoptosis (93).

Butyrate has several beneficial effects in human body. In this study we mainly focused the role of some butyric acid derivatives in colorectal cancer prevention. There are several studies have detected the anti colorectal cancer activity of butyrate and its derivatives. Most of the studies have shown the anti cancer activity of butyrate is due to

its histone deacetylase inhibition. Very few studies have determined the role of GPR109A receptor in CRC prevention. But here we focuses the anti-cancer activity of some selected butyric acid derivatives through GPR109A receptor and its binding affinity with this receptor.

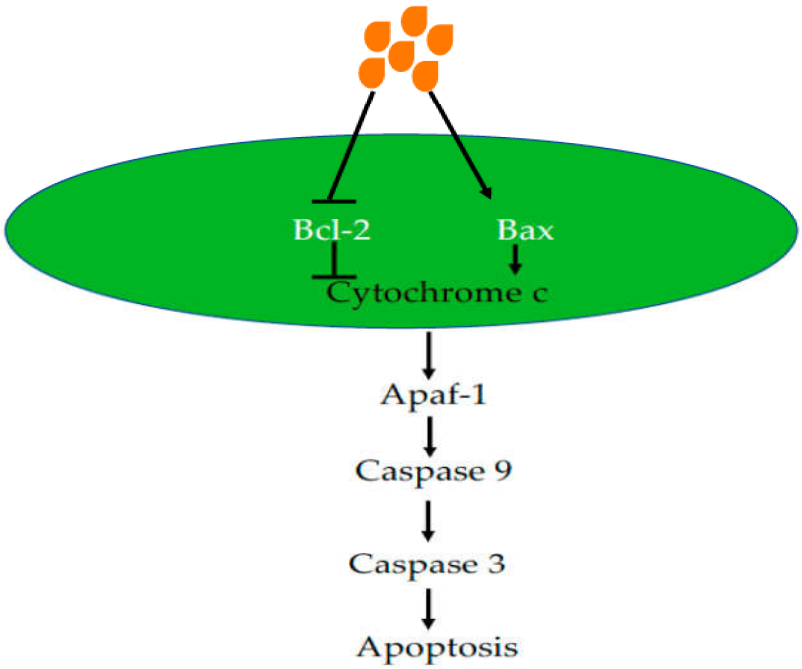


Figure 1.6: Figure illustrates the role of butyrate in mitochondrial apoptotic pathway (93)

1.9. Gap areas

The diet derived bioactive compound butyrate has several beneficial roles in colon. Butyrate is readily used by normal colonocytes for energy metabolism, but for cancer cells it serves as an

inhibitor of proliferation. The short half-life of butyrate considerably restricts its therapeutic application. Some derivatives like 4-phenyl butyric acid and trybutyrins have tested in colon cancer cell lines. But here we mainly focus on the non-toxic stable natural derivatives of butyric acid. So we need to check its effect on colon cancer cells. Butyric acid is preferred in cancer treatment due to its toxic effect in cancer cells and its beneficial role in non-cancerous cells. More over butyric acid act as the ligand for GPR109A receptor. Some studies suggest that the anti-cancerous properties of butyric acid are mainly through this receptor. Considering this aspects in this study, we focused to analyse the binding affinity of some butyric acid derivatives with GPR109A receptor using computer aided screening. However, the experimentally well determined crystal structure of the receptor is not available for evaluating the computer aided interaction studies, the best alternative method is to construct a three dimensional model of the target protein.

Hence, the present study mainly focused to analyse the binding efficiency of selected butyric acid derivatives against GPR109A receptor, a probable drug target of colorectal cancer, by computational modelling and also evaluate the *in vitro* effects of these derivatives on human colon cancer cell proliferation.

Dietary fiber mediated colon cancer prevention is mainly attained through butyrate. The anti-cancer effect of butyric acid has been extensively studied in various kinds of colorectal cancer cells. The review mainly focused the anti-carcinogenic effect of butyric acid in colon cancer cells and various kinds of apoptotic mechanisms behind this anti-cancer activity.

2.1. Anti cancer effect of butyric acid in colorectal cancer cells

Butyric acid is an abundant short chain fatty acid and plays an important role in the maintenance of colon homeostasis. Butyrate mediates cancer cell growth inhibition and differentiation through its histone deacetylase inhibitory action. Butyrate also exerts its anti-cancerous activity by changing cellular metabolism through the regulation of metabolic enzymes. During the carcinogenesis process most of the cells exhibits irregular metabolism which characterised by an increased rate of glycolytic pathway results in high lactate production. This effect is known as Warburg effect and is the major feature of cancerous cells. Besides this, other pathways are also alters the tumoregenesis process. Glutamine, an essential amino acid exceeds in cancerous cells than other amino acids. The catabolism of glutamine provide the carbon source for the precursor of tricarboxylic acid cycle (TCA) intermediates (80).

The cells that treated with butyrate utilizes it as energy source rather than glucose. So the highly glycolytic colon cancer cells switch into butyrate utilizing phenotype. Butyrate enhances the gene expression of pyruvate dehydrogenase kinase and leads the hyperacetylation of histone. Butyrate act as the major energy source for colonocytes. But cancerous cells utilizes glucose as its major

energy substrates. The highest rate of glycolysis enhances the lactate production and thereby enhances proliferation of the cells. Tumour cells also consumes large amount of glutamine. Glutamine also enter into citric acid cycle and converted to pyruvate. Glutamine can also be converted to lactate, if the malate is converted to pyruvate in cytoplasm and this pathway produce NADPH which act as the energy source for fatty acid synthesis. Butyrate has a capacity to reduce lactate production. Butyrate alters the glutamine utilization in cancerous cells and thereby unable to incorporate the carbon derived from pyruvate to lipid. Instead glutamine derived carbon incorporated into the lipid and decrease in lactate production observed in butyrate treated cancerous cells (80).

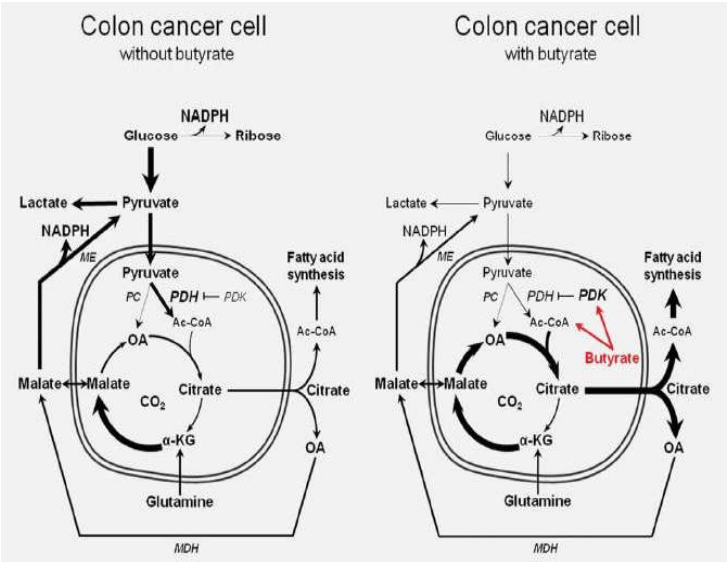


Figure 2.1: Figure illustrate the metabolism of butyrate in cancerous colon cells. The highest rate of catabolism of glutamine in cancerous cells enhances the synthesis of lactate. The presence of butyrate induces the incorporation of carbon derived from glutamine to lipids and a decrease in lactate production occurs. Butyrate modulated glutamine metabolism is strongly associated with pyruvate dehydrogenase complex (PDC) , an inhibitor of Pyruvate dehydrogenase kinase (PDK) (80)

Butyric acid plays an important role in the development of intestinal barrier functions. According to Peng et al, the treatment of Caco-2 colon cancer cells with butyrate enhances the activity of AMP-activated protein kinase (AMPK). AMPK involved in the regulation of tight junction in intestine (94). One of the feature of colorectal cancer with microsatellite instability (MSI) related to the intestinal barrier is the frequent secretion of mucins, MUC2 and MUC5AC (95). Treatment of colon cancer cell line LS174T with butyrate enhances the stimulation of MUC2 production (96)

Butyrate exerts its protective effects in colon cancer cells by activating biotransformation enzymes. Glutathione-s-transferases (GST) is such an enzyme which reduces endogenous as well as exogenous carcinogens and thereby blocking cancer initiation. The mechanism of action of GST is mainly by the conjugation of electrophilic intermediate of carcinogens with Glutathione. Butyrate enhances the activity of GST by up regulating its protein expression (97).

Studies suggested that the highest concentration of butyrate in the large bowel increases the apoptosis rate in colon cancer cells (98). Butyrate induces colon cancer cell apoptosis by activating JNK MAP kinase pathway. MAP (mitogen activated protein) kinase pathway involved in the regulation of cell proliferation through the extra cellular signal regulated proteins like JNK (C-Jun N-terminal kinase). JNK mainly involved in the regulation of apoptosis and cell growth arrest. Butyrate effectively induces the cytotoxic effect in colon cancer cells characterised by DNA fragmentation as well as the activation of

caspase-3 and 9 (79). Studies shows that the treatment of colon cancer cells with butyrate induces apoptosis within 24 hours by activating caspase-3 (99). JNK mediated apoptosis in colon cancer cells involves the stimulation of Bax gene(83).

Butyrate inactivates cox-2 expression in colon cancer cells through its histone deacetylase inhibitory action. Cox-2 is actively involved in colon carcinogenesis and its activity increases approximately 25 fold by TNF-α. The histone deacetylase inhibitory action of butyrate block the expression of TNF-α activation of cox-2 protein in HT-29 colon cancer cells (100). Butyrate actively suppresses the colon carcinogenesis by mediating various kinds of regulatory mechanisms. It reduces the expression of *OPN* (Osteopontin) and *COX-2* (cyclooxygenase) mRNA in primary colon tissue. *OPN* and *COX-2* are the tumour promoting genes and are overexpressed in colon cancer cells (101).

Butyrate induced apoptosis of colorectal cancer cells are depends on the hyper activation of Wnt/beta-catenin pathway (102). Sodium butyrates act as anti-cancer agent in colon carcinoma cells by re expressing the ASC protein. ASC is the Apoptosis associated spec like protein , is a pro-apoptotic signalling factor silenced in tumour cases (103). P_{21} acts as an effector of butyrate induced apoptosis in colorectal cancer cells. P_{21} is a cyclin CDK-inhibitor exert cell cycle arrest at G_1 phase. The induction of P_{21} expression and growth arrest of colon cancer cells by butyrate is mediated through histone hyperacetylation (104). Calcium butyrate is significantly induces anti-

proliferative activity in colon cancer cells *invivo* as well as *invitro* (105).

Butyrate induces apoptosis in colon cancer cells through various mechanisms. It suppresses the activity of Neuropilin 1 (NRP-1) by inhibiting the transcription factor Sp1 (specificity protein 1) trans activation to inhibit the angiogenesis and metastasis. Neuropilin is a transmembrane receptor for VEGF (Vascular endothelial growth factor) and is up regulated in cancer cells (106). Butyrate promote the apoptosis of colorectal cancer by up regulating Wnt signalling pathway (102). Another mechanism for the prevention of colorectal cancer is the regulation of microRNA expression. It has been reported that sodium butyrate up regulate the expression of miR-203 and inhibit the cell proliferation cell invasion and colony formation (107). The histone hyperacetylation activity of butyrate induces P^{21wafl} and there by mediates apoptosis in colon cancer cells P^{21wafl} is the Cyclin dependent kinase inhibitor and is associated with cell cycle arrest (104). The microRNA miR-92a is over expressed in human colon cancer tissue than normal cells. The treatment with butyrate reduces the level of precursor of this miRNA and by reducing c-myc and thus enhances p57 level. The regulatory gene c-myc is the proto oncogenic gene constitutively expressed in cancer cells. p57, cyclin dependent kinase inhibitor is a tumour suppressor gene. It causes cell cycle arrest in G_1 phase (108).

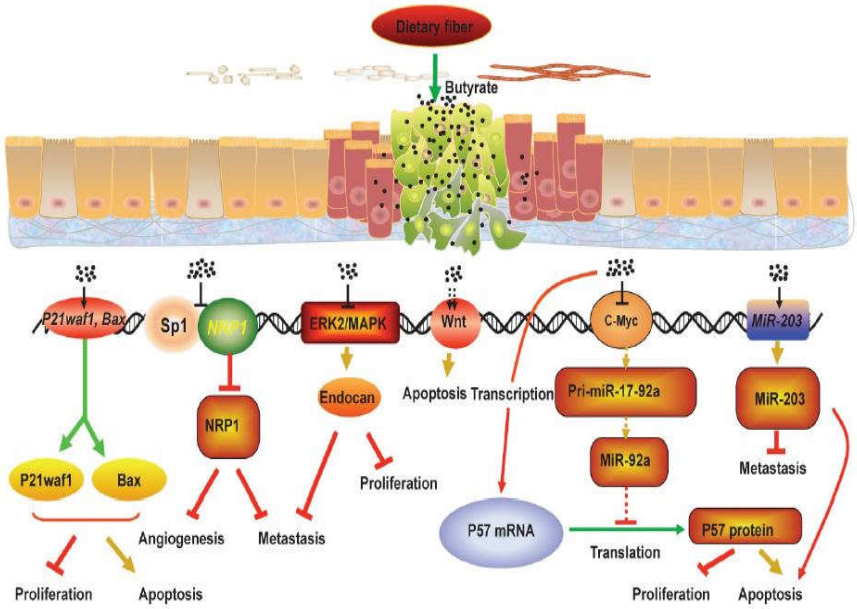


Figure 2.2: Figure illustrates the mechanisms of suppression of tumour development by butyrate. The major mechanisms involved in tumour suppression are decrease in the activity of NRP-1, up regulation of Wnt signalling pathway, up regulation of the expression of miR-203 micro RNA and the activation of p57 (109).

2.2. Role of GPR109A in butyrate induced apoptosis in colon carcinogenesis

GPR109A (HCA_2) was first identified in 2001, as a transcript induced in Interferon-γ (IFN- γ) treated murine macrophages and the receptor also termed PUMA-G (Protein up-regulated in macrophages by IFN- γ) (110).

GPR109A was discovered as a high affinity receptor for Nicotinic acid (91). The identification of this receptor leads to the

molecular mechanism behind the dyslipidemic effect of nicotinic acid. Because the receptor is expressed in adipocytes and its activation causes the inactivation of hormone sensitive lipase and reduces lipolysis. The plasma concentration of nicotinate is not sufficient to activate the receptor. Hence the ketone body in the blood, β-hydroxybutyrate was identified as the ligand for GPR109A receptor (111). The receptor is not only expressed in adipocytes, but also expressed in other tissues like skin, hepatocytes, retinal cells, bones, intestinal epithelial cells and immune cells like dendritic cells and macrophages (112, 113). The expression of the receptor is relatively high in colonic epithelial cells and lumen facing apical membrane of intestine because of the presence of butyrate. Gut micro-biota plays a major role in regulating the receptor expression. In germ free mice, the expression of the receptor reduced, but after re-colonization expression come back (55).

Activation of gpr109a receptor inhibit the adenylyl cyclase activity which results reduction in cAMP level. cAMP mediate the prolipolytic stimuli β-adrenoceptor (β-AR) which stimulate the activation of cAMP in cellular level and leads the activation of protein kinase A (PKA). Subsequently the lipolysis occur through the various proteins, including hormone-sensitive lipase (HSL) and Adipocyte triglyceride lipase (ATGL) which results in the reduction of free fatty acid (FFA). Low level of free fatty acid results in shortage of substrate for triglyceride synthesis in hepatic cells. As a result plasma level of VLDL and LDL drops and decreased exchange occur between

cholesterol esters carried by HDL and triglycerides in LDL and VLDL. Subsequently increase in plasma HDL concentration occur (112).

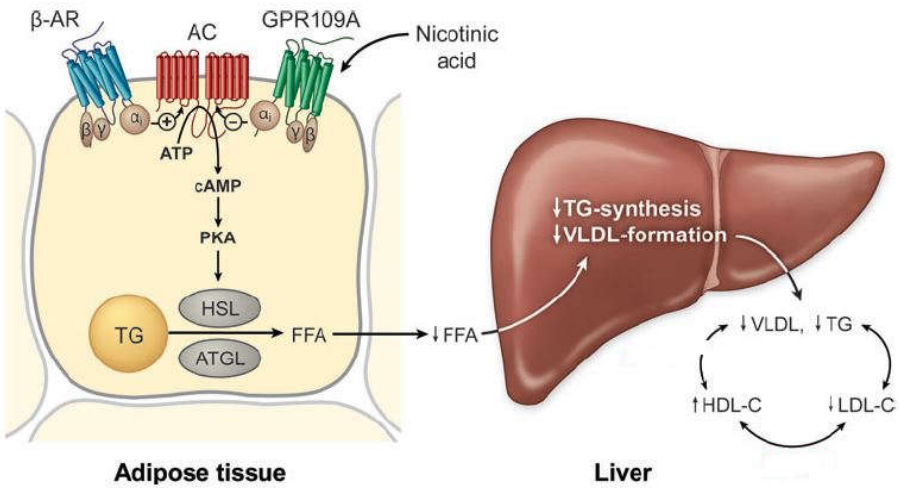


Figure 2.3: Figure illustrates the lipid lowering effect of GPR109A in adipocytes and liver. The mechanisms involves the reduction in cAMP level. cAMP mediate the prolipolytic stimuli β-adrenoceptor (β-AR) which stimulate the activation of cAMP in cellular level and leads the activation of protein kinase A (PKA). Subsequently the reduction of free fatty acid (FFA) occur. Low level of free fatty acid results in shortage of substrate for triglyceride synthesis in hepatic cells and decreased exchange occur between cholesterol esters carried by HDL and triglycerides in LDL and VLDL and increase in plasma HDL concentration occur (112)

Butyric acid shows low affinity with the G-protein coupled receptor GPR109A. The actual ligand of this receptor is Nicotinate. This receptor is expressed in intestinal epitheial cells as well as the lumen facing apical membrane of colonic cells. The receptor is silenced in the case of colon cancer in humans. The silencing of the receptor in colon cancerous cells are mainly due to the DNA hyper methylation. The re expression of the receptor induces apoptosis in

colon cancer cells in the presence of its ligands Nicotinate or butyrate. The receptor induced apoptosis in colon cancer cells are does not depends on the histone deacetylase inhibitory action of butyrate (58).

G-protein coupled receptor GPR43 is act as the receptor for short chain fatty acids. This receptor is expressed in large intestine as well as hematopoietic tissues. This receptor is also silenced in colon cancer cells. The treatment with short chain fatty acids, especially bytyrate and propionate restore the expression of GPR43 in Human colonic adenocarcinoma cells HCT8 by inducing G_0/G_1 cell cycle arrest and caspases activation (114).

Experimentally well determined structure of GPR109A is not available. Very few studies are there associated with the receptor structure. GPR109A (HM74A) and GPR109B (HM74) are structurally homologous (115). The site directed mutagenesis study of ligand binding residues combined with generation of chimeric receptors with GPR109A and GPR109B identified that Asn86/Trp91 TMH (Trans membrane helix) and 2 ECL (Extra cellular loop). The amino acid residues Arg111 (TMH3), Ser178 (ECL2), Phe276 (TMH7) and Tyr284 (TMH7) are present in the critical binding site of Nicotinic acid (116).

2.3. Butyric acid and its derivatives in cancer treatment

One of the major health benefit of butyric acid is its anti-cancer activity. It inhibits the growth of tumour cells effectively and has less adverse effects in clinical trials. But the rapid uptake and metabolism limits its chemotherapeutic action (117). So further studies are focused

in its alternatives. Tributyrin is a rapidly absorbed derivative of butyric acid effectively inhibit the growth of cancerous cells than natural butyrate (118). Studies shows that the tributyrin emulsions have anti-carcinogenic effect in HT-29 cells. Such an emulsion is more stable and can be used in drug delivery system (119). Tributyrin not only exerts its growth inhibitory effects in colon cancer cells, it also has inhibitory effects in other type of cancers like prostate cancer. So it can be used in advanced cancer treatment or the recurrence after specific therapy (120).

Another derivative, 4-phenyl butyric acid shows growth inhibitory effects in gastric cancer cells associated with cell cycle arrest (121). Calcium butyrate exhibits anti-inflammatory as well as anti-tumour property invivo as well as invitro (105). 3-n-butyrate, a stable derivative of butyric acid has long half life in blood, approximately 30-40 times more stable than butyrate. The combination of 3-n butyrate with 5-Fluorouracil, chemotherapeutic agent used to treat colorectal cancer inhibit the growth of colon cancer tumours invivo. The combination of 5-Fluorouracil and 3-n butyrate shows synergetic effect in colon cancer cells (122).

The present study focused to analyse the effects of some selected naturally occurring non-toxic derivatives of butyric acid in colorectal cancer cells *invitro* and their binding potential towards GPR109A receptor by computer aided screening.

OBJECTIVES

1. To evaluate the anti-proliferative activity of butyric acid derivatives in colon cancer cell lines.

2. To investigate the butyric acid derivatives induced apoptotic mechanisms in colon cancer cell lines.

3. To analyse the binding potential of butyric acid derivatives towards GPR109A receptor by *insilico* methods.

Materials

Human colorectal carcinoma cell lines, HCT116 were obtained from National Centre for Cell Sciences, Pune, India. 25cm^2 Cornings cell culture flasks, RPMI-1640 media and 0.25% Trypsin- EDTA and Antibiotic-antimycotic solutions were purchased from Sigma-Aldrich, Darmstadt, Germany. Fetal bovine serum, Sodium butyrate, Tributyrin, 5-Fluorouracil and MTT were purchased from Himedia, India. Indole-3-butyric acid was purchased from Fischer Scientific, Mumbai, India. 2-Amino-n-butyric acid was purchased from Sisco Research Laboratories Pvt.Ltd, Mumbai, India. Nicotinate was purchased from S D Fine-Chem Limited, Maharashtra, India. Caspase-3 chromogenic substrate was purchased from Sigma-Aldrich. All other reagents and chemicals used were analytical grade.

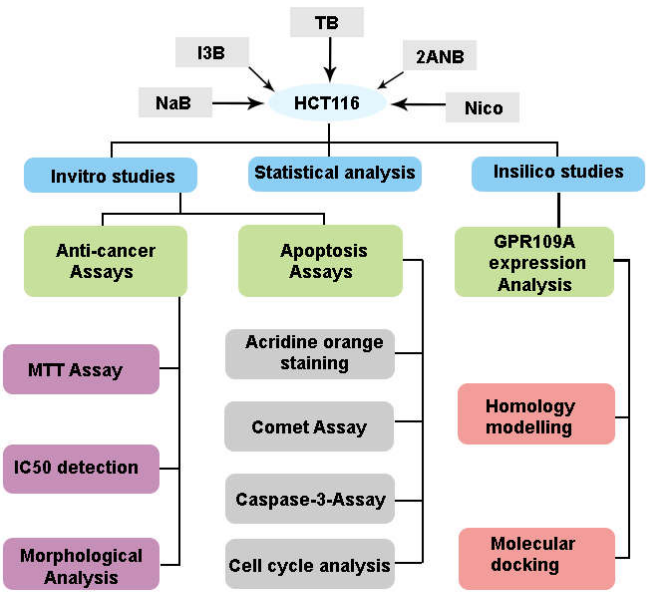


Figure 3.1: Diagram summarising the overall work. NaB: Sodium butyrate, I3B: Indole-3-butyric acid, TB: Tributyrin, 2ANB: 2-Amino-n-butyric acid, Nico: Nicotinate

3.1. Invitro studies

3.1.1. Determination of Anti-proliferative activity of butyric acid derivatives in colorectal cancer cells

3.1.1.1. Cell culture

Human colorectal carcinoma cell lines, HCT116 were obtained from National centre for Cell Sciences, Pune. Cells were cultured in RPMI1640 media (Sigma) supplemented with 10% Fetal Bovine Serum (FBS) and antibiotic-antimycotic solution (1 ml/100 ml) in a humidified atmosphere at 37ºC with 5% CO_2(123).

For sub culturingthe cells, checked the cell culture flask to ensure the cells are in confluent stage and the spent media was discarded. Then the cells were washed twice with Phosphate buffered saline (PBS: 137 mM NaCl, 2.7 mM KCl, 8 mM Na_2HPO_4 and 2 mM KH_2PO_4, P^H: 7.4) (0.2ml/cm^2) to remove traces of serum that inactivate trypsin. 2 ml Trypsin-EDTA solution (0.1 ml/ cm^2) was added to the flask and kept for 5-10 min for the detachments of the cells. Then added fresh culture media to inactivate the trypsin in the cell suspension. Centrifuge the suspension for 5 min at 1800 rpm and discarded the supernatant and added fresh media. A 1/10 aliquot of the cell suspension was placed into a new flask with the cell culture medium required for that flask size. Cell culture flasks were then placed to the CO_2 incubator. After 24h, the culture was checked to ensure that cells are reattached and the pH of the medium is approximately 7.4. Medium is then changed necessary until the next subculture. Viable cells were counted using trypan blue dye by

haemocytometer. For counting, the cells were mixed with equal volume of 0.4% trypan blue dye and kept at 5min at room temperature. Healthy cells exclude the dye, where as damaged cells absorbed the dye. Cells were frozen and stored at -80ºC. For the preservation, cell suspension was diluted to 1:1 with freezing medium (RPMI media with 20% FBS and antibiotic-antimycotic solution (1 ml/100 ml) and 5% Dimethyl Sulfoxide (DMSO) used as the cryoprotectant) (123, 124).

3.1.1.2. MTT Assay

The derivatives used for the study were sodium butyrate, indole-3-butyric acid, tributyrin and 2-amino-n-butyric acid. The cell proliferation was measured using MTT (3-(4, 5-dimethyl thiazol-2-yl)-2, 5-diphenyl tetrazolium bromide) colorimetric assay. 3.5×10^4 cells were seeded into 96 well plates. After 24 hours of plating, the cells were treated with sodium butyrate, indole-3-butyric acid, tributyrin, 2-amino-n-butyric acid and nicotinic acid at a final concentration of 1 mM, 5 mM and 10 mM for 24, 48 and 72 hours. 10 ul of 3-(4,5 Dimethylthiazol-2-yl)2,5-Diphenyl tetrazolium bromide (MTT, Stock: 2 g/L in Phosphate buffered saline) solution added into each of 96 wells. The cells were incubated at 37ºC for 4 hours, the medium was removed and 100 µl of Dimethyl sulfoxide (DMSO) was added to solubilise the formazan. The micro plate was shaken and the optical density values were measured at 540 nm using plate reader (121, 125). The relative inhibition rate was calculated as a percentage as follows:

Relative inhibition rate : 1- (Absorbance $_{Sample}$ /Absorbance $_{control}$) X 100%

The stock solutions of indole-3-butyric acid and Tributyrin were prepared in dimethyl sulfoxide (DMSO) and diluted to 1 mM, 5 mM and 10 mM respectively for cell culture treatment (Final concentration of the DMSO was maintained at 1% in culture). Sodium butyrate, nicotinate, 2-amino-n-butyric acid and 5-fluorouracil were prepared in phosphate buffered saline and diluted in RPMI media to a final concentration of 1 mM, 5 mM and 10 mM. in this study, 5-Fluorouracil used as the positive control for the treatment and Nicotinate used as the GPR109A receptor ligand. Ten possible combinations (Sodium butyrate + Nicotinate, Indole-3-butyric acid + Nicotinate, Tributyrin + Nicotinate, Indole-3-butyric acid + 2-amino-n-butyric acid, 2-amino-n-butyric acid + Nicotinate, Tributyrin + 2-amino-n-butyric acid, sodium butyrate + indole-3-butyric acid, sodium butyrate + tributyrin, sodium butyrate + 2-amino-n butyric acid and indole-3-butyric acid + tributyrin) of the compounds (Each compounds with 5 mM concentration) were also tested.

3.1.1.3. Determination of IC_{50} values

IC_{50} values of Sodium butyrate, Indole-3-butyric acid, Tributyrin, 2-Amino-n-butyric acid and Nicotinate were determined using MTT assay. Treatment was done with increasing concentrations (1-10 mM) of butyric acid derivatives, Nicotinate and 5-Fluorouracil for 24, 48 and 72 hours (121, 126).

3.1.1.4. Morphological analysis

HCT116 cells were treated with IC_{50} concentration of butyric acid derivatives, nicotinate and 5-Fluorouracil for 24 hours and morphological changes in HCT116 cells were examined by inverted microscope (Leica EC3).

3.1.2. Analysis of butyric acid derivatives induced apoptotic mechanism in colorectal cancer cells

3.1.2.1. Acridine orange staining

To perform the morphological analysis of cell nuclei, Acridine orange staining was done. 8.825×10^5 HCT 116 cells were seeded in Cell culture flasks. Cells were treated with IC_{50} concentrations of Sodium butyrate, Tributyrin, Indole-3-butyric acid, 2-Amino-n-butyric acid, Nicotinate and 5-Fluorouracil for 24 hours. After 24 hours treatment, cells were harvested and resuspended in Phosphate buffered saline (PBS). Then the cells were stained with 5µg/ml Acridine orange in PBS and observed under Fluorescent microscope (Leica DM6 B) (125).

3.1.2.2. Comet Assay

To analyse the DNA damage, comet assay was done. HCT116 cells were treated with IC_{50} concentration of butyric acid derivatives as well as Nicotinate for 24 hours. The cells were harvested by trypsinization. 100 µl of 0.5% normal melting agarose in PBS was dropped onto a micro slide, covered immediately with a cover slip, and then placed at 4˚C for 10 min. The cover slip was removed after the

gel had set. 50 µl of cell suspension was mixed with 50 µl of 1% low melting agarose and applied on top of the gel, coated over the micro slide and covered with a cover slip, and then placed at 4˚C for 10 min. The cover slip was again removed after the gel had set. A third coating of 50 µl of 0.5% low melting agarose was placed on the gel and allowed to set at 4˚C for 15 min. After solidification of the agarose, the cover slips were removed, and the slides were i mMersed 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 hours. The slides, after removed from the lysis solution, were placed horizontally in an electrophoresis chamber. The reservoirs were filled with an electrophoresis buffer (300 mM NaOH, 1.2 mM EDTA) until the slides were just immersed in it, and the DNA was allowed to unwind for 30 min in electrophoresis solution. Then the electrophoresis was carried out at 25 V and 300 mA for 20 min. After electrophoresis, 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) (127).

3.1.2.3. Caspase-3 Assay

The assay of Caspase-3 activity was performed using the colorimetric method. This assay was based on the detection of the amount of Ac-DEVD-p-NA substrate cleaved by cell lysates to release the coloured 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 hours. Following treatment,

the cells were washed in PBS and suspended in lysis buffer (50 mM HEPES, P^H 7.4, 5 mM Triton X100, 5 mM DTT) for 15 minute. Lysed cells were centrifuged at 16,000 x g, 4ºC for 15 minutes. Protein concentrations in lysate were determined using the Bradford assay. For the assay, to each tube containing 0.07 mM substrate in assay buffer (20 mM HEPES, P^H 7.4, 0.1% Triton X100,5 mM DTT, 2 mM EDTA) was added to 10-60 µl of cell lysate, bringing the total volume of each well to 100µl. Caspase-3 activity was assessed by measuring the optical density at 405nm using AM 2100 Micro plate Reader (ALERE). Activity expressed as µmol p-NA released per minute per milligram of protein (99, 128, 129).

3.1.2.4. Cell cycle analysis

For analysing the cell cycle distribution, HCT 116 cells (100000 cells/ml) were treated with IC_{50} concentration of Indole-3-butyric acid and Tributyrin. Camptothecin (15 µM) used as the standard for the treatment. The cells were fixed in cold 70% ethanol and stored at 4ºC. Cells were incubated in Propidium iodide/RNase staining buffer and cell cycle was analysed using BD FACS calibur (121). PI histogram of the gated Cell singlets distinguished cells at the Sub G_0/G_1, G_0/G_1, S, and G_2/M cycle phases. Gating of cell cycle phases is approximate and can be refined using software (Cell Quest Software, Version 6.0) analysis.

3.2. Analysis of the expression of GPR109A receptor by *insilico* method

3.2.1. Homology modelling of GPR109A receptor

The target sequence of GPR109A was retrieved from UniProt KB databases with the accession number Q8TDS4. The hypothetical model of GPR109A receptor was predicted by homology modelling by Modeller 9.18 (130). The best homologous templates were retrieved by BLAST (Basic Local Alignment Search Tool) search based on the parameters like total score, E-value, query coverage and percentage of identity and similarity. The best homologous templates used for the present study were, human P2y1 receptor in complex with Bptu (PDB ID: 4XNV, chain A), Crystal structure of active mu-opioid receptor bound to the agonist Bu72 (PDB ID: 5C1M, chain A), Crystal structure of human angiotensin receptor in complex with inverse agonist Olmesartan (PDB ID: 4ZUD, chain A) and XFEL structure of human angiotensin receptor (PDB ID: 4YAY, chain A). The description of the selected template was tabulated in **Table 3.1**.

From the alignment, 3D model containing all non-hydrogen atoms were obtained automatically using the method implemented in Modeller 9.18. The secondary structure of hypothetical model was predicted by Stride web server. The hypothetical model was visualised by UCSF Chimera (131).

Table 3.1. The best homologous template of GPR109A (UniProt ID: Q8TDS4) retrieved from BLAST search for comparative modelling.

PDB ID	Description	Organism	Max Score	Query coverage (%)	Max identity (%)	E Value	Length	Resolution (Å)
4XNV_A	Chain A, The Human P2y1 Receptor In Complex With Bptu	Unknown	120	54	34	2e-30	421	2.2 Å
5C1M_A	Chain A, Crystal Structure Of Active Mu-opioid Receptor Bound To The Agonist Bu72	*Mus musculus*	93.6	73	30	3e-21	296	2.1 Å
4ZUD_A	Chain A, Crystal Structure Of Human Angiotensin Receptor In Complex With Inverse Agonist Olmesartan At 2.8a Resolution.	Unknown	91.3	83	23	5e-20	410	2.8 Å
4YAY_A	Chain A, XFEL structure of human Angiotensin Receptor	*Homo sapiens*	92	85	23	3e-20	414	2.9 Å

3.2.2. Model Evaluation

The modelled protein was evaluated by various bioinformatics tools. The predicted model was energy minimized by ModRefiner (132). The structural alignment and super imposition was carried out by Superpose server and the root mean square deviation (RMSD) between the target and template were estimated. The stereo-chemical quality of the hypothetical model was checked by PROCHECK (133) and the model was further evaluated by Verify-3D (134). The overall quality of modelled protein with an error function plot was computed by ERRAT (135). The structural comparison of the theoretical model with the experimental model was predicted by ProSA and the Z-score was determined (136).

3.2.3. Molecular docking

The receptor-ligand interactions were predicted by molecular docking. The ligands used in this study were retrieved from PubChem and ChemSpider databases. The ligands were retrieved in the .sdf format and converted to the PDB format by Open Babel (137). The binding site of the hypothetical model was analysed by Pocket Finder and the grid boxes were generated to cover the entire active site using autogrid with suitable 3D dimensions. Molecular docking was performed via flexible docking approach by AutoDock vina (138). The best docked poses were analysed by various parameters such as the binding energy,

cluster RMS, number of hydrogen bonds, other weak interactions, and interacting residues within 1.Å cavity .

3.3. Statistical analysis

The experimental protocols were replicated as independent trails and variations in the results were tested for statistical significance. The data were presented as mean ± standard deviation. All measurements and analysis were carried out from six wells per treatment from three independent experiments. One way analysis of Variance (ANOVA) with post hoc test, Duncan on SPSS 21 (Statistical Package for the Social Sciences) software was used to estimate the level of significance between means at 95% confidence interval ($p<0.05$).

4.1 Effect of butyric acid derivatives on the proliferation of HCT 116 cell lines

There are many chemotherapeutic agents are used for the treatment of cancer. This includes anti-metabolites, anti-tumour antibiotics, alkylating agents, enzymes and hormones. There is no proper medication that destroys only cancerous cells without damaging the normal cells. Most of the drugs cause dangerous side effects (139). As a lifestyle dependent disease, colorectal cancer incidence can be reduced by changing lifestyle and dietary factors. Gut bacteria and dietary fiber plays an important role in the maintenance of colonic health. The production of butyric acid by the dietary fiber fermentation through the intestinal bacteria is mainly responsible for these effects (140).

In the present study anti-proliferative activities of some selected butyric acid derivatives were carried out against human colorectal cancer cell line, HCT116. Sodium butyrate, Indole-3-butyric acid, Tributyrin and 2-Amino-n-butyric acid were the derivatives used for the study. Nicotinate used as the GPR109A receptor ligand and 5-Fluorouracil used as the positive control for the treatment.

The cell viability assay was performed to evaluate the percentage of cytotoxicity of butyric acid derivatives and nicotinate in HCT116 cells. After exposure to butyric acid derivatives and nicotinate for 24, 48 and 72 hours, the growth of HCT 116 cells were significantly inhibited (1, 5 and 10 mM for 24 and 48 hours, 1 and 5 mM for 72 hours, p <0.05 and

10 mM for 72 hours, $p<0.01$). Tributyrin shows highest inhibition rate at 24 hours , 1, 5 and 10 mM concentration. Sodium butyrate shows a two fold increase in the inhibition rate at 24 hours, 10 mM concentration than 5 mM concentartion. The inhibitory effects were suggestive to be dose and time dependent. There were no significant differences between tributyrin and 5-fluorouracil observed for 72 hours at 10 mM concentration **(Figure 4.1)**.

The present study showed that the butyric acid derivatives and nicotinate have a time and dose dependent effect on the proliferation of HCT116 cell lines as shown in previous studies which utilised Sodium butyrate (81). There were few studies which revealed that the higher doses of sodium butyrate (5 and 10 mM concentration) induces apoptosis in colon cancer cell lines (125, 141). A study suggests that tributyrin, a triglyceride analogue of butyrate can act as a potent anti-cancer agent against melanoma. Tributyrin emulsion suppressed the melanoma cells in dose dependent manner (142). Tributyrin consist of 3 butyric acids esterified to a glycerol. It can be cleaved by intracellular lipases and esterase into 3 molecule of butyric acid. The butyrate concentration in plasma is about 0.5 mM. Hence, the minimum effective concentration can be achieved by oral administration of tributyrin (143). The presence of indole ring gave more stability to butyrate. The stability study suggests that the indole-3-carboxaldehyde derivatives were stable for more than 24 hours under physiological conditions (144). Thus, the indole-3-butyric acid can be act as a stable drug.

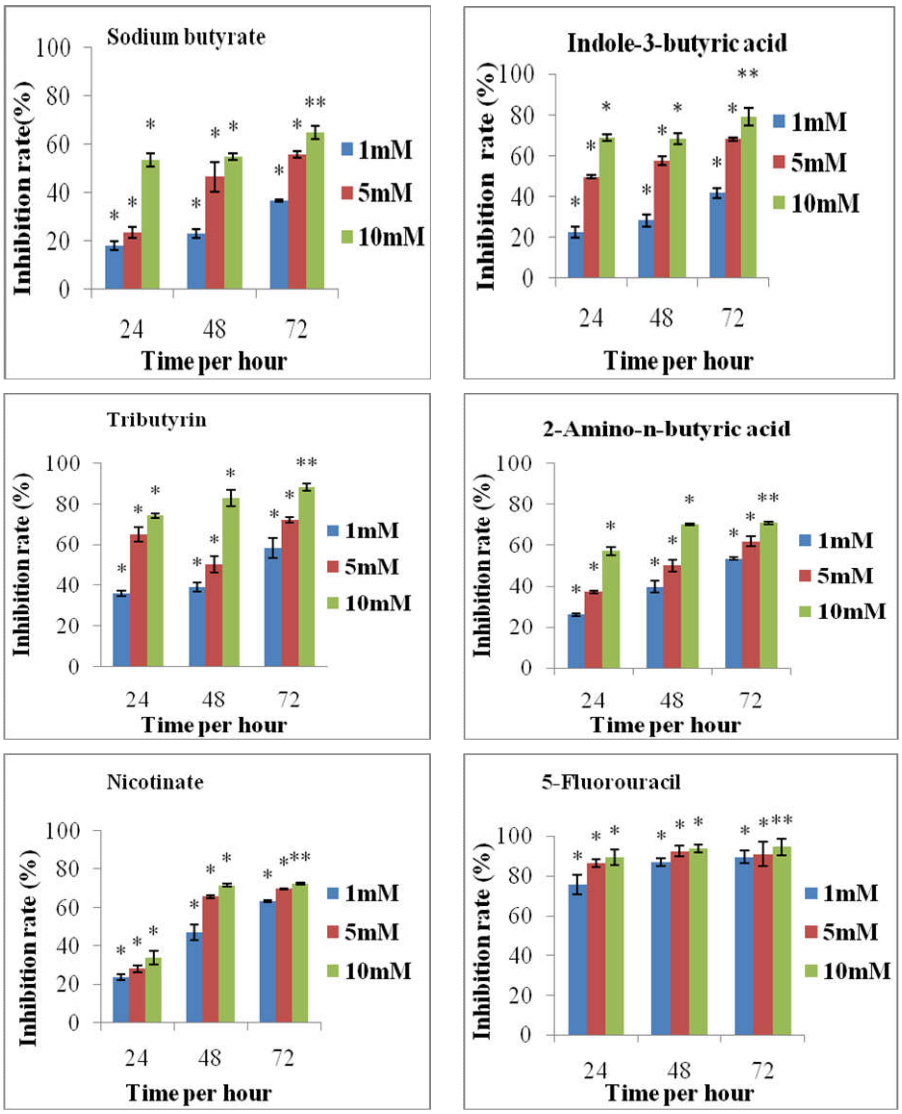


Figure 4.1: The figure illustrates the inhibitory effect of Sodium butyrate, Indole-3-butyric acid, Tributyrin, 2-Amino-n-butyricacid, Nicotinate and 5-Fluorouracil on HCT116 cell. HCT116 cells were treated with 1 mM, 5 mM, and 10 mM concentrations for 24, 48 and 72 hours. Inhibition rate was measured by MTT assay. Data were from 6 wells per treatment from 3 independent experiments. The results represents the Mean ± Standard deviation , $^{*}p$ <0.05 (1, 5 and 10 mM for 24 and 48 hours, 1 and 5 mM for 72 hours) and $^{**}p$ <0.01(10 mM for 72 hours) determined by one way ANOVA with posthoc test Duncan.

Table 4.1: The percentage of inhibitory rate of HCT 116 cells treated with butyric acid derivatives at 24 hours.

Test compounds	Inhibition rate at 24 hours (%)		
	1 mM	5 mM	10 mM
Sodium butyrate	18.14±1.72	23.47±2.14	53.72±2.74
Indole-3-butyric acid	22.8±2.65	49.81±0.80	68.98±1.76
Tributyrin	35.95±1.30	65.06±3.61	74.30±1.23
Nicotinate	23.91±1.57	28.18±1.80	33.93±3.36
2-Amino-n- butyric acid	26.06±0.66	37.11±0.73	57.18±1.90
5-Fluorouracil	75.57±5.04	86.42±1.75	89.31±3.96

Table 4.2: The percentage of inhibitory rate of HCT 116 cells treated with butyric acid derivatives at 48 hours.

Test compounds	Inhibition rate at 48 hours (%)		
	1 mM	5 mM	10 mM
Sodium butyrate	23±1.69	46.51±6.07	54.87±1.37
Indole-3-butyric acid	28.34±2.89	57.65±2.18	68.35±2.70
Tributyrin	39.02±2.22	50.21±3.97	83.01±4.17
Nicotinate	47.36±4.09	65.74±0.79	71.79±0.84
2-Amino-n- butyric acid	39.58±2.91	50.15±2.85	70.32±0.51
5-Fluorouracil	86.92±1.81	92.4±2.60	93.71±1.77

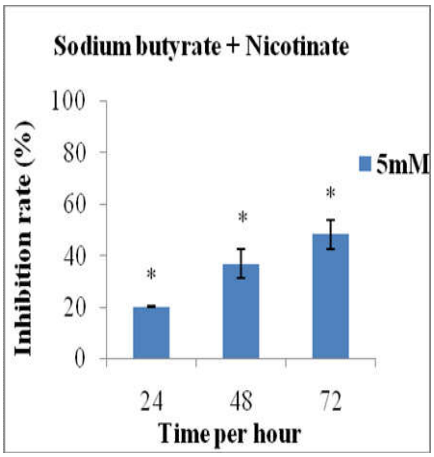
Table 4.3: The percentage of inhibitory rate of HCT 116 cells treated with butyric acid derivatives at 72 hours.

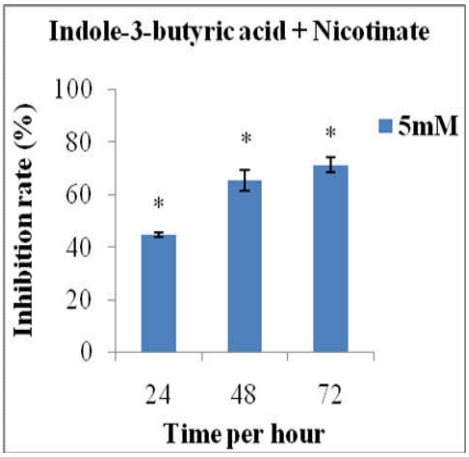
Test compounds	Inhibition rate at 72 hours (%)		
	1 mM	5 mM	10 mM
Sodium butyrate	36.62±0.58	55.85±1.47	64.73±2.66
Indole-3-butyric acid	41.83±2.44	68.29±0.96	79.3±4.40
Tributyrin	58.39±5.09	72.2±1.47	88.38±1.88
Nicotinate	63.3±0.39	69.94±0.25	72.52±0.51
2-Amino-n- butyric acid	53.47±0.79	61.86±2.46	70.87±0.81
5-Fluorouracil	89.43±3.18	90.96±6.01	94.56±4.21

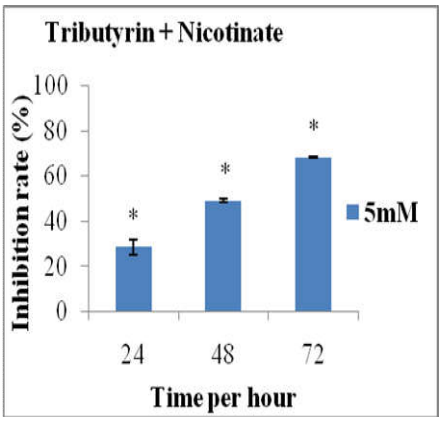
4.2. Effect of combinations of the butyric acid derivatives on the proliferation of the HCT116 cell lines

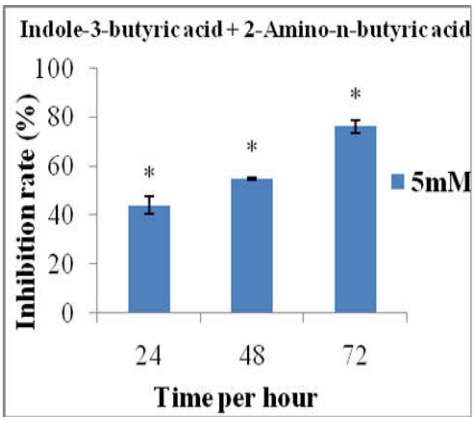
To further elucidate specifically which combinations of butyric acid derivatives induce more inhibition rate, ten possible combinations were used. At 24 hours, Indole-3-butyric acid and Tributyrin combination shows antagonistic effect and the combinations of Sodium butyrate, 2-Amino-n-butyric acid and Nicotinate with Indole-3-butyric acid and Tributyrin shows synergestic effects. At 48 hours, combination of Indole-3-butyric acid and Tributyrin shows synergestic effect **(Figure 4.2)**. However, 5-fluorouracil and 3-n-butyrate showed synergestic antitumour activity against human colorectal cancers (122). The current study depicted that the combinations of Sodium butyrate and tributyrin (24 hours), indole-3-butyric acid and tributyrin (48 hours), indole-3-butyric acid and nicotinate, sodium butyrate and nicotinate, indole-3-

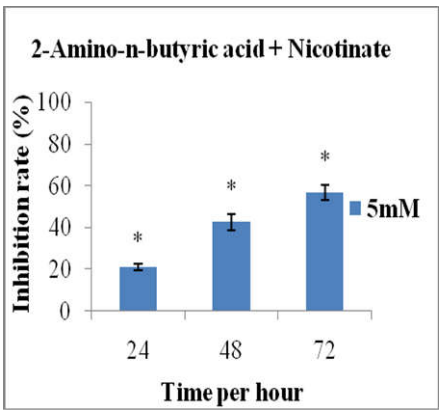
butyric acid and tributyrin and indole-3-butyric acid and 2-amino-n-butyric acid (72 hours) were maximally induced cytotoxicity.

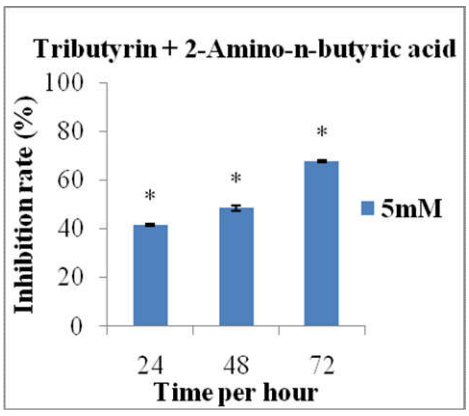












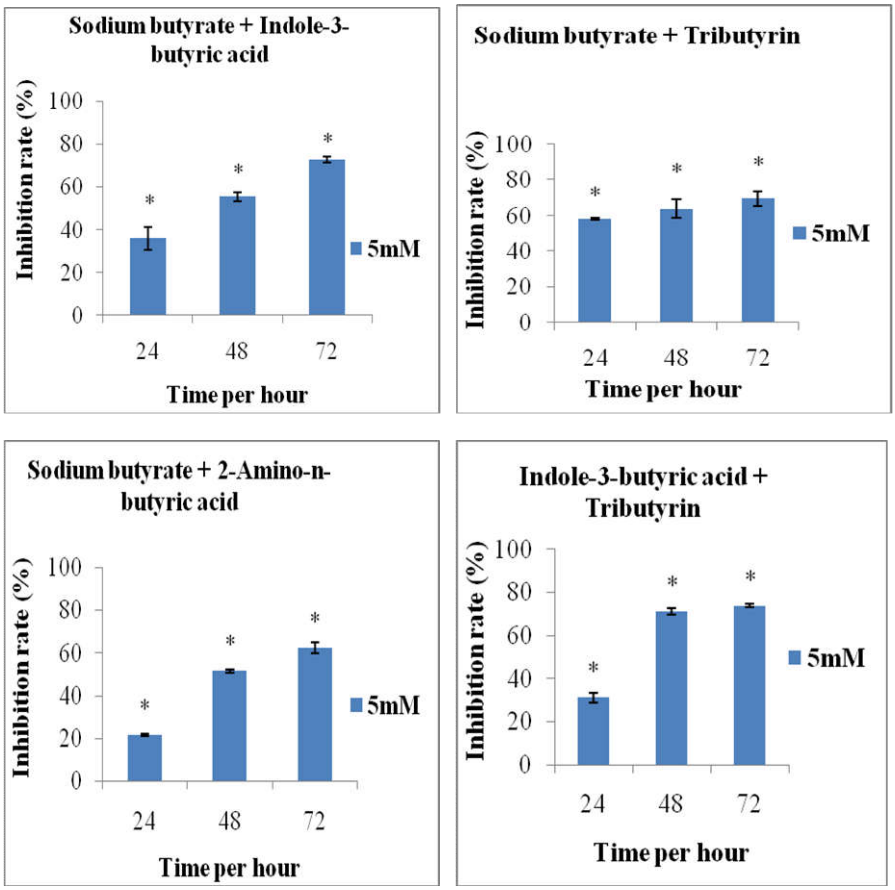


Fig 4.2 : The figure illustrates the inhibitory effect of various combinations of butyric acid derivatives in HCT116 cells. The combinations are Sodium butyrate and Nicotinate, Indoe-3-butyrate and Nicotinate, Tributyrin and Nicotinate, Indole-3-butyrate and 2-Amino-n-butyric acid, 2-Amino-n-butyric acid and Nicotinate, Tributyrin and 2-Amino-n-butyric acid, Sodium butyrate and Indole-3-butyric acid, Sodium butyrate and Tributyrin, Sodium butyrate and 2-Amino-n-butyric acid, Indole-3-butyric acid and Tributyrin on HCT 116 cells. Each compounds with 5 mM concentration for 24, 48 and 72 hours MTT assay was conducted. Data were expressed as Mean ± Standard deviation in 6 wells per treatment from three independent experiments. $^{*}p$ <0.05 considered statistically significant.

Table 4.4: The percentage of inhibitory rate of HCT 116 cells treated with combinations of butyric acid derivatives.

Test compounds	Inhibition rate (%)		
	24 hours	48 hours	72 hours
Sodium butyrate + Nicotinate	20.18±0.21	36.8±5.57	49.27±5.61
Indole-3-butyric acid + Nicotinate	44.57±0.99	65.39±3.90	71.3±2.85
Tributyrin + Nicotinate	28.45±3.24	48.99±0.81	68.11±0.27
Indole-3-butyric acid + 2-Amino-n-butyricacid	44.09±3.61	54.87±0.47	76.22±2.82
2-Amino-n-butyric acid + Nicotinate	21.1±1.70	42.66±3.83	56.57±3.49
Tributyrin + 2-Amino-n-butyric acid	41.36±0.39	48.41±0.95	67.74±0.46
Sodium butyrate + Indole-3-butyric acid	35.89±5.46	55.38±2.19	72.57±1.32
Sodium butyrate + Tributyrin	58.15±0.77	63.76±5.33	69.51±4.17
Sodium butyrate + 2-Amino-n-butyric acid	21.6±0.60	51.47±0.73	62.5±2.49
Indole-3-butyric acid + Tributyrin	31.23±2.23	70.98±1.49	73.79±0.63

4.3. 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 shows least IC_{50} value at 24 hours (6.28±0.10, 4.94±0.19 mM respectively). There is no significant difference between the IC_{50} values of Indole-3-butyric acid and Tributyrin at 48 hours (5.84±0.16 and 5.01±0.38) and 72 hours (4.39±0.25 and 3.30±0.22). The IC_{50} values of butyric acid derivatives as well as Nicotinate were shown in **Table 4.5**.

Compared with other derivatives, the IC_{50} value of Nicotinate was highest at 24 hours (15.13±1.34). This result suggests that, as a ligand the butyric acid derivatives are more efficient in its cytotoxic action in cancer cells than nicotinate. There is no significant difference between the IC_{50} value of Indole-3-butyric acid, Tributyrin, 2-Amino-n-butyric acid and Nicotinate at 48 hours treatment. At 72 hours, the least IC_{50} values are observed in Tributyrin, Nicotinate and Indole-3-butyric acid (3.30±0.22, 3.71±0.03, 4.39±0.25) treated cells. The derivatives, Tributyrin and Indole-3-butyric acid induced cytotoxic effect in HCT116 cells with an IC_{50} of 4.94±0.19 and 6.28±0.10 respectively at 24 hours. This is the least IC_{50} value showed by the derivatives at 24 hours. An earlier study demonstrated that the cytotoxic induction of tributyrin emulsion in melanoma with an IC_{50} of ~2 mM (142).

Table 4.5: The IC_{50} values of butyric acid derivatives at 24, 48 and 72 hours.

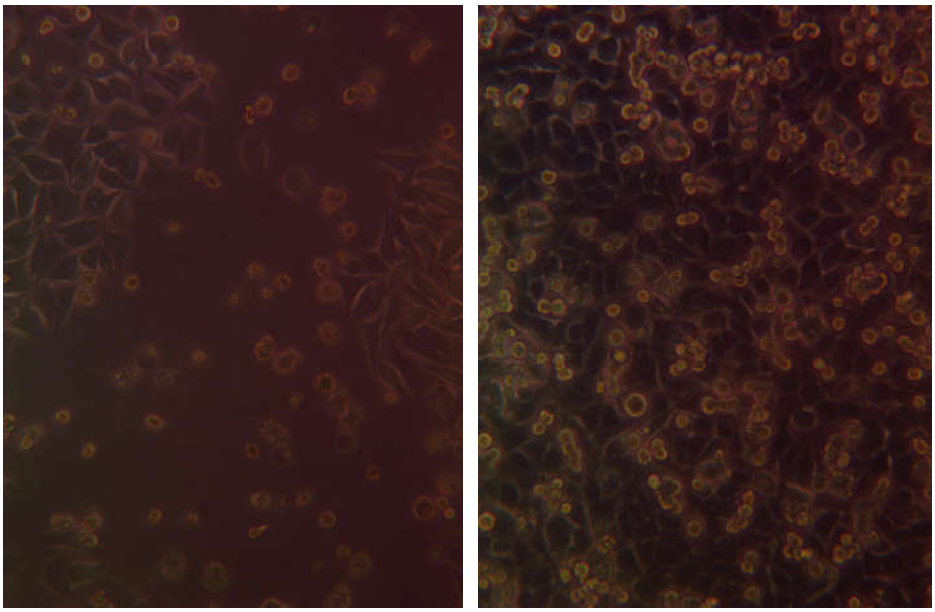
Test compounds	IC_{50} Values (mM)		
	24 hours	**48 hours**	**72 hours**
Sodium butyrate	9.53±0.55	7.83±0.46	5.98±0.12
Tributyrin	4.94±0.19	5.01±0.38	3.30±0.22
Indole-3-butyric acid	6.28±0.10	5.84±0.16	4.39±0.25
2 Amino-n- butyric acid	8.09±0.21	5.75±0.02	4.65±0.10
Nicotinate	15.13±1.34	4.65±0.21	3.71±0.03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

4.4. Morphological analysis

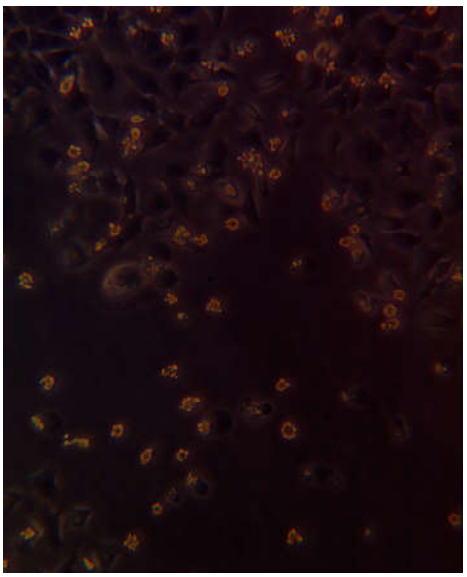
Inorder to determine the role of apoptosis in cell growth inhibition by butyric acid derivatives, morphological changes in

HCT116 cells after treatment with IC_{50} concentration of butyric acid, were examined by inverted microscope (Leica EC3). The result showed that the butyrate treated cell lines showed low cell confluence, an indicative of apoptosis (**Figure 4.3**). Moreover, the nicotinate treated cells are floating in nature, indicated that nicotinate treatment resulted in reduced adherence. Untreated or DMSO control cells were attached to the culture plates with greater than 90% confluent under the same condition as those for butyric acid derivatives as well as nicotinate treated cells.

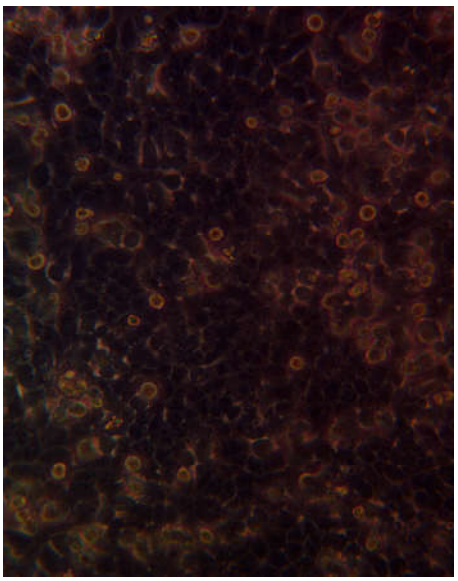
Sodium butyrate Treated cells **PBS control cells**



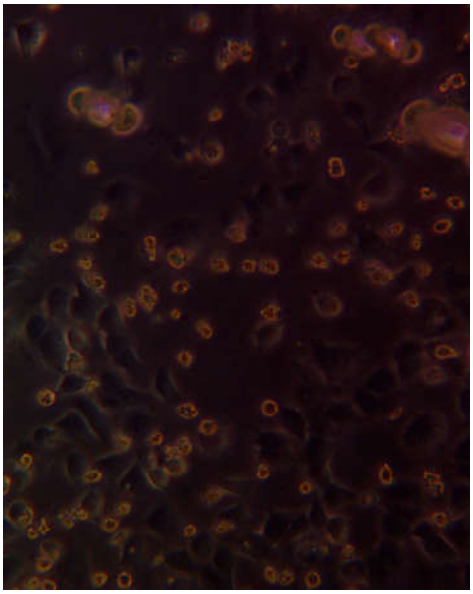
Indole-3-butyric acid treated cells



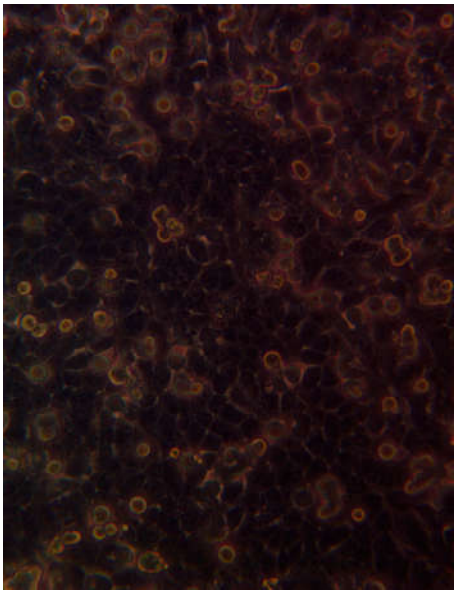
DMSO control cells



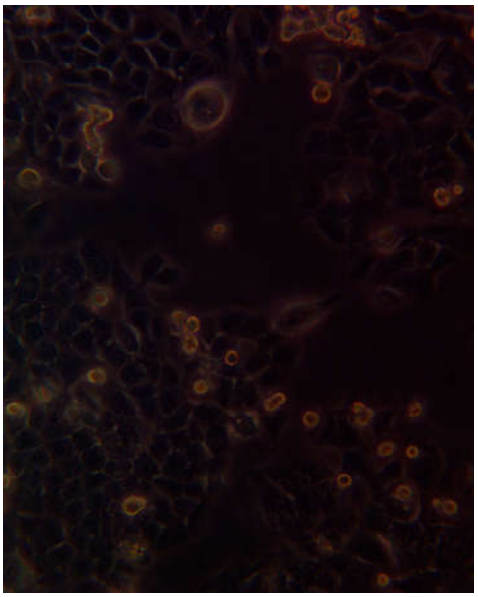
Tributyrin treated cells



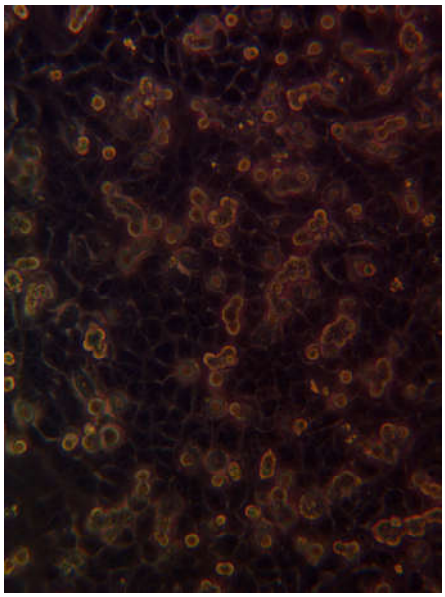
DMSO control cells



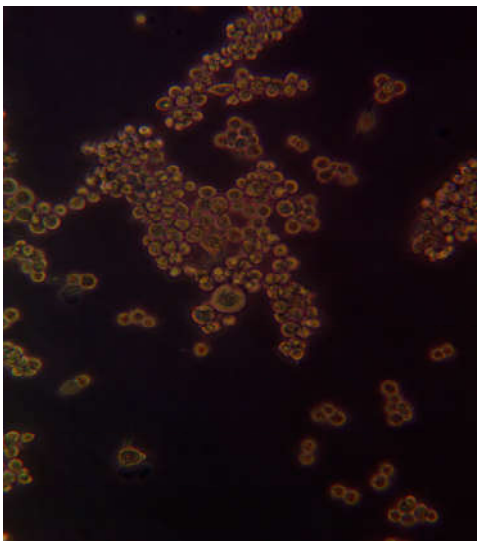
2-Amino-n-butyric acid treated cells



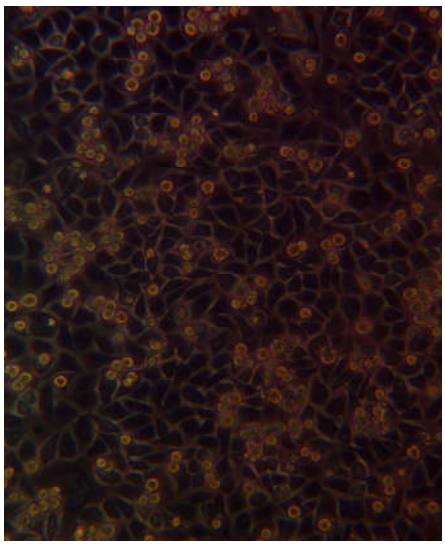
PBS control cells



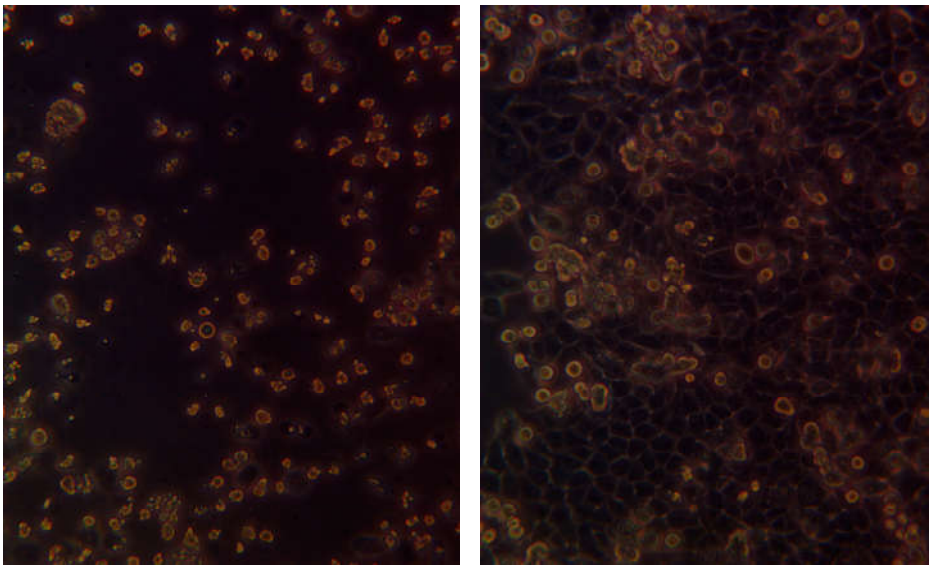
Nicotinate treated cells



PBS control cells



5-Fluorouracil treated cells **PBS control cells**



Untreated cells

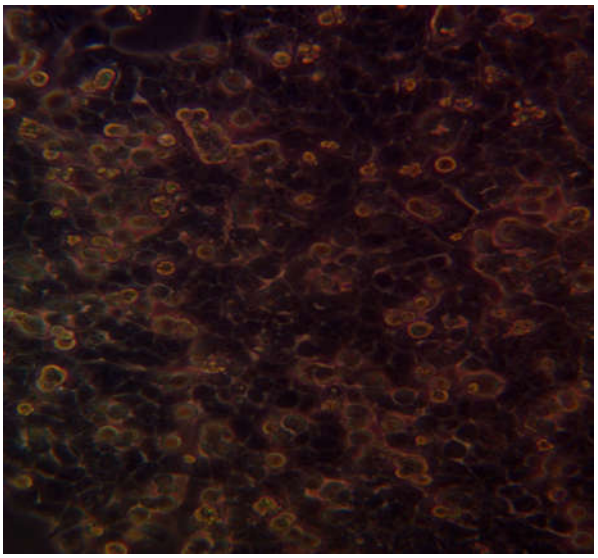


Figure 4.3: The morphology of HCT116 cells treated with IC_{50} concentrations of Sodium butyrate, Indole-3-butyric acid, Tributyrin, 2-Amino-n-butyric acid, Nicotinate, 5-Fluorouracil and untreated control cells. Images were observed at 20X magnification.

In the present study we mainly focuses the role of butyric acid derivatives as anti-colorectal cancer agents. The compounds used in the present study shows several beneficial effects in cancer prevention. Butyric acid has short half life and is rapidly cleared from the plasma. The short half life restricts its therapeutic application. The studies suggested that the half life of sodium butyrate was only 6.1 min in plasma. But , tributyrin resides in plasma more than sodium butyrate (145, 146). Some studies show the growth inhibitory effects of nicotinate in colon cancer cells. Curcumin nicotinate effectively induces HCT 116 cells growth through p53 mediated apoptosis and cell cycle arrest (147). Indole derivatives show potential anti-carcinogenic effects in variety of cancerous cells. Indole regulate cell signal transduction, cell proliferation, apoptosis and angiogenesis (148). From the anti-proliferative assay it can be concluded that butyric acid derivatives (sodium butyrate, indole-3-butyric acid, tributyrin and 2-amino-n-butyric acid) as well as nicotinate effectively inhibits HCT116 cell proliferation.

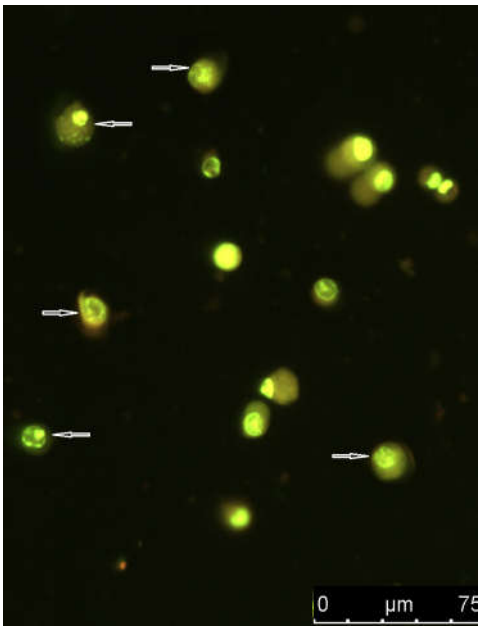
In this study, we investigated the mechanisms of reaction that triggers apoptosis in HCT116 cells treated with butyric acid derivatives. The anti-proliferative and apoptotic activity of shortchain fatty acids, especially butyric acid is mainly due to its histone hyperacetylation (149). The derivatives used for the study are, Sodium butyrate, Indole-3-butyric acid, Tributyrin and 2-Amino-n-butyric acid. Besides these derivatives, the effect of Nicotinate on HCT116 cell was also tested, because it acts as a ligand for GPR109A receptor protein. Studies shows that the receptor is highly expressed in normal human colon tissue, but is silenced in colon carcinoma cells. The silencing of the receptor is mainly due to its methylation of promoter DNA (150). Nicotinate as well as butyrate suppresses the colon cancer in a GPR109A dependent manner (151).

5.1. Morphological assessment of apoptotic cells by Acridine Orange staining

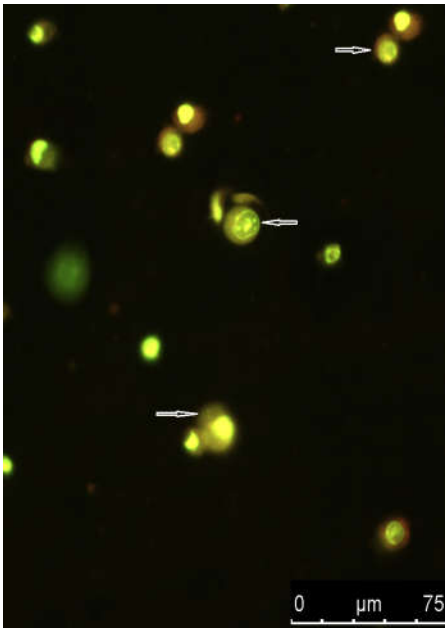
To investigate the involvement of apoptosis in butyric acid treated cells, morphological analysis using acridine orange staining was done. Acridine orange is one of the most reliable methods for analysing cellular apoptosis. The cellular changes of apoptosis are, cell shrinkage, masses of condensed chromatin and membrane blebbing (152). The fluorescent images reveal the apoptosis inducing effects of these compounds in HCT116 cells (**Figure 5.1**).The result of morphological analysis of cells using acridine orange showed that the untreated cells showed normal configuration with green nuclei without apoptosis. Cells treated with IC_{50} concentration of butyric acid derivatives as well as nicotinate showed early apoptosis features

including membrane blebbing and nuclear fragmentation and this morphological features of apoptosis are comparable with the cells that treated with 5-Fluorouracil, the positive control of the test. The Detached population of cells treated with butyric acid derivatives showed the typical morphological characteristics of apoptosis such as membrane blebbing and fragmented nuclei compared with untreated control.

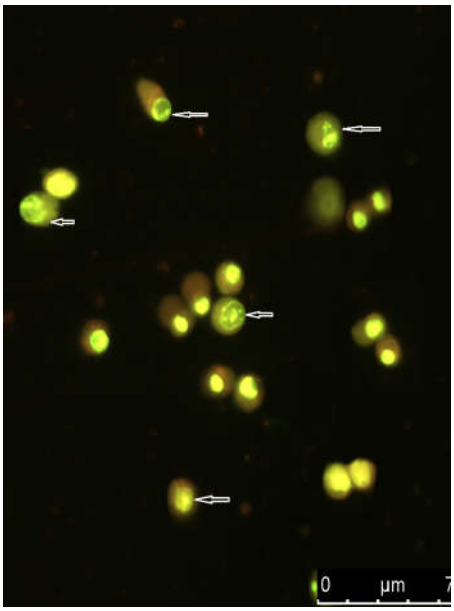
Sodium butyrate treated cell



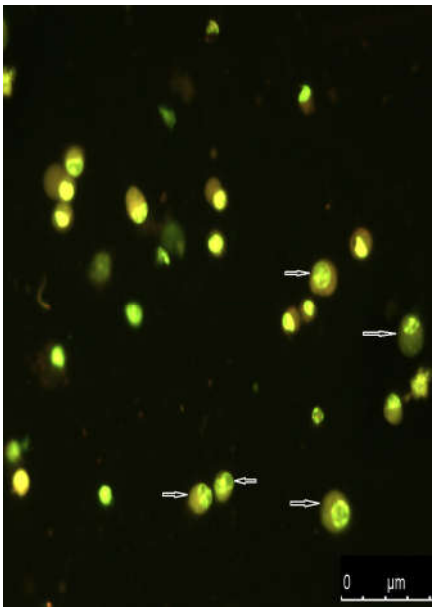
Indole-3-butyric acid treated cells



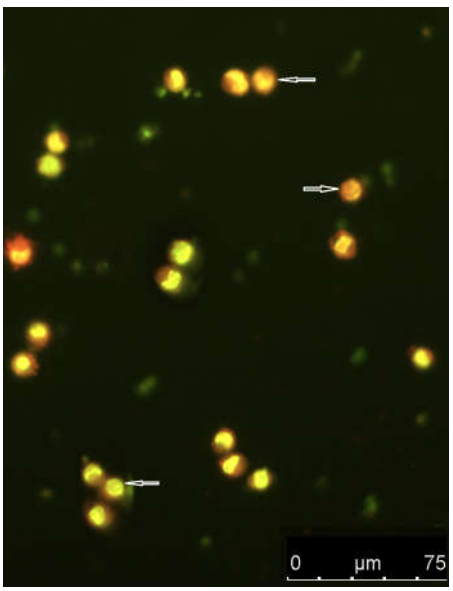
Tributyrin treated cells



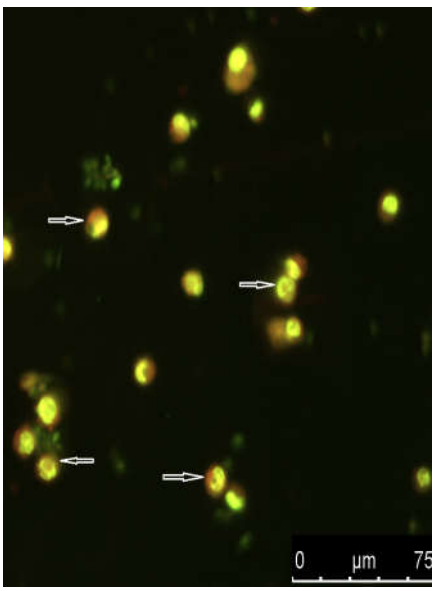
2-Amino-n-butyric acid treated cells



Nicotinate treated cells



5-Fluorouracil treated cells



Untreated Control

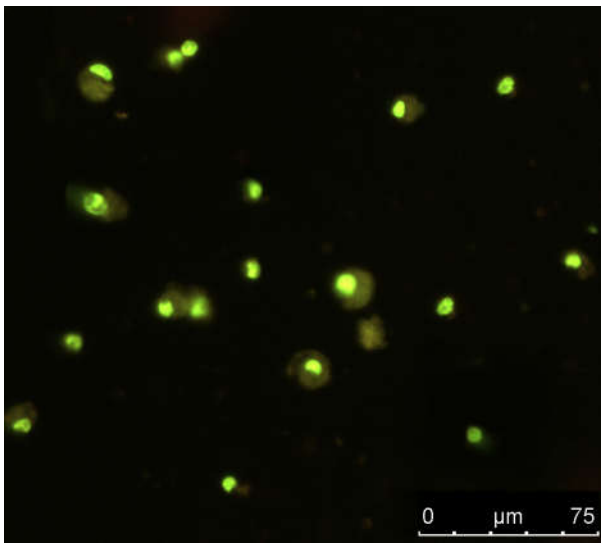
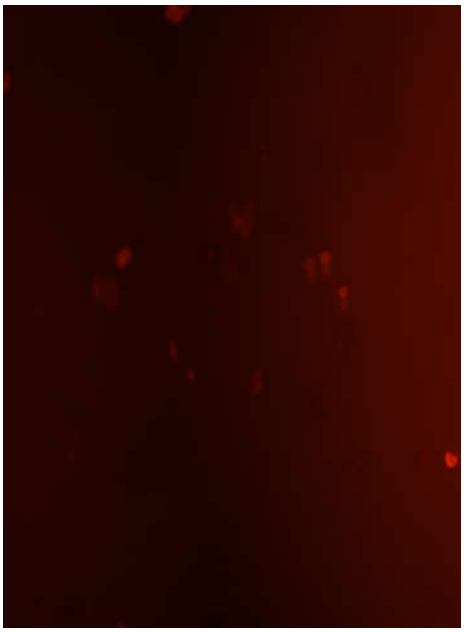


Figure 5.1: HCT 116 cells stained with acridine orange after treatment with IC_{50} values of Sodium butyrate, Indole-3-butyric acid, Tributyrin, 2-Amino-n-butyric acid, Nicotinate, 5-Fluorouracil and Untreated control.

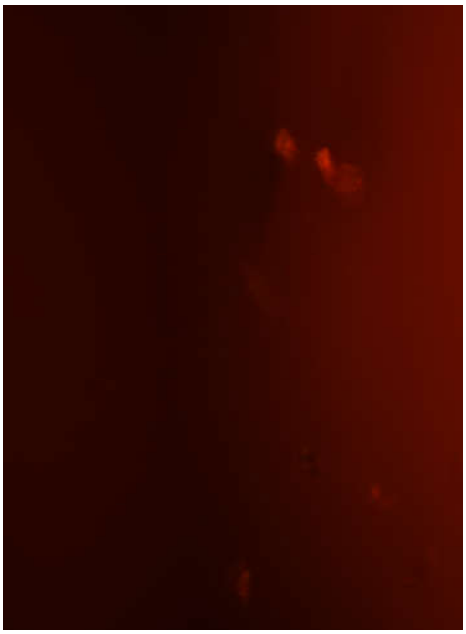
5.2. Analysis of DNA damage by the Comet Assay

The DNA damage of butyric acid derivatives treated cells were analysed using the technique of single-cell gel electrophoresis (comet assay) in agarose gel matrix. DNA damage is another hallmark of apoptosis. After treating the cells with butyric acid derivatives, nicotinate and 5-Fluorouracil, comet assay was done to determine the DNA damage. Well-formed comets were observed in the cells treated with Indole-3-butyric acid, Tributyrin and Nicotinate under fluorescent microscope. The result is similar to those found in positive control, 5-Fluorouracil treated cells. There is no comet like structures are found in untreated control (**Figure 5.2)**.

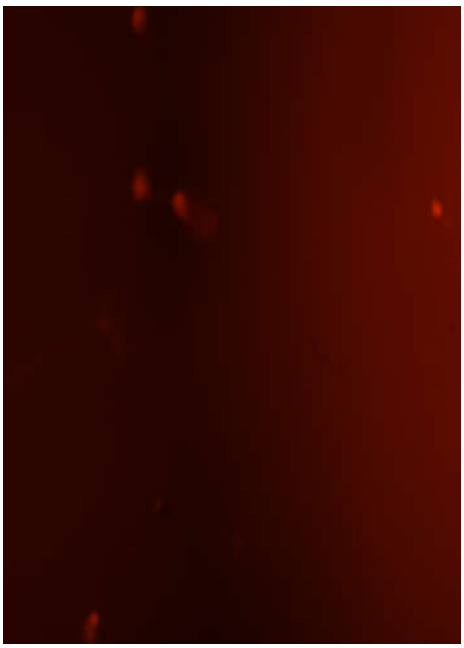
Sodium butyrate treated cells



Indole-3-butyric acid treated cells



Tributyrin treated cells



2-Amino-n-butyric acid treated cells

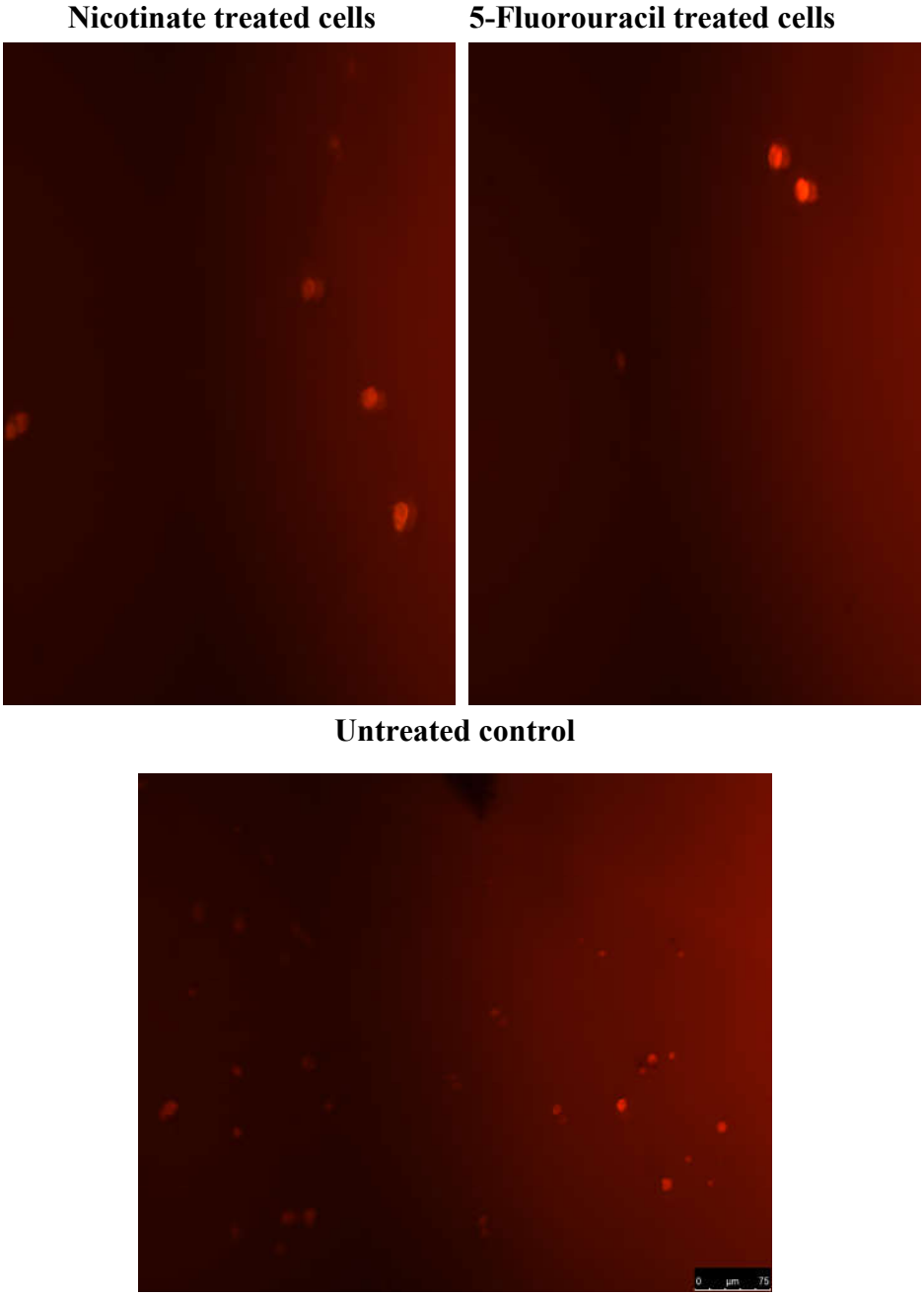


Figure 5.2: Comet assay of HCT 116 cells treated with IC_{50} concentrations of Sodium butyrate, Indole-3-butyric acid, Tributyrin, 2-Amino-n-butyric acid, 5-Fluorouracil and Untreated control.

5.3. Caspase -3 assay

To investigate the Caspase-3 activity, HCT116 cells were treated with the IC_{50} concentrations of butyric acid derivatives as well as Nicotinate for 24 hours. The result of this experiment shows that treatment of HCT116 cells with butyric acid derivatives as well as Nicotinate significantly activate caspase-3 compared with untreated control. The activity of caspase-3 in HCT 116 cells treated with butyric acid derivatives as well as nicotinate is shown in **Table 5.1**. There is no significant difference between the caspase-3 activity of Butyric acid derivatives, Nicotinate and 5-Fluorouracil (**Figure 5.3**).

It was observed that apoptosis occurred in derivatives treated HCT116 cells via caspase-3 mediated signalling. The activity of caspase-3 in Sodium butyrate, Indole-3-butyric acid, Tributyrin, 2-Amino-n-butyric acid, nicotinate and 5-Fluorrouracil in U/mg of protein are 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 shows an activity of 1.98±0.49 U/mg of protein.

Caspase-3 is a frequently activated protease in mammalian cell apoptosis (153). The activation in colon cancer cell line is depends on peroxisome proliferator-activated receptor (154). The previous studies suggest that Butyrate induces apoptosis by caspase-3 activation in various kind of colon cancer cell lines and is mediated via upregulation of pro apoptotic BAK and inducing caspase-3 mediated cleavage of poly ADP ribose polymerase (PARP) (155, 156). So the results

suggest that butyric acid derivatives induces caspase-3 dependent apoptotic pathway in HCT116 cells.

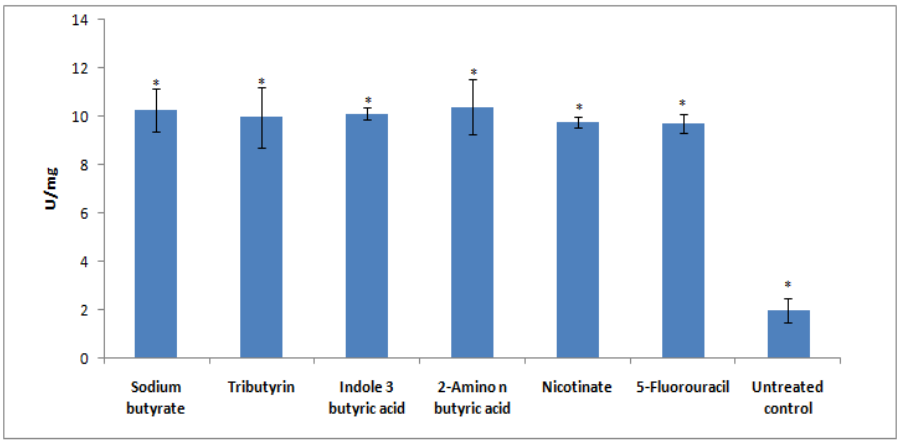


Figure 5.3: Activity of Caspase-3 in HCT 116 cells after treating with IC_{50} concentration of butyric acid derivatives, Nicotinate and 5-Fluorouracil at 24 hours. Data were presented as Mean ± Standard deviation in 6 wells per treatment from 3 independent experiments. *P<.05 considered statistically significant.

Table 5.1: Table shows the Caspase-3 activity (U/mg) of HCT116 cells treated with IC_{50} concentration of butyric acid derivatives, Nicotinate and 5-Fluorouracil. 1 U=µmol/min/mg of protein

Test compounds	Caspase-3 Activity (U/mg)
Sodium butyrate	10.28±0.9
Indole-3-butyric acid	10.13±0.23
Tributyrin	9.98±1.25
2-Amino-n-butyric acid	10.4±1.13
Nicotinate	9.76±0.22
5-Fluorouracil	9.71±0.4
Untreated control	1.98±0.49

5.4. Effect of Cell cycle distribution of Indole-3-butyric acid and Tributyrin on HCT116 cells

Cell cycle analysis was done to determine the effect of butyric acid derivatives on the cell cycle progression of HCT116 cells. The derivatives, Indole-3-butyric acid and Tributyrin shows least IC_{50} value at 24 hours. So the cell cycle distribution of these two derivatives were analysed by flow cytometry. The DNA content histogram is shown in **Figure 5.4**. The result of cell cycle analysis suggested that in Sub G_0/G_1 phase 4.82% and 16.95%, in G_0/G_1 phase 58.14% and 51.15%, in S phase, 8.17% and 11.18 % , in G_2/M phase 20.91% and 17.07% of cell population were present in Indole-3-butyric acid and Tributyrin treated cells respectively (**Table 5.2**). The increase in the Sub G_0/G_1 population indicate the presence of apoptotic cells. Previous study showed that butyrate causes G_1 arrest in cancer cell lines (157).

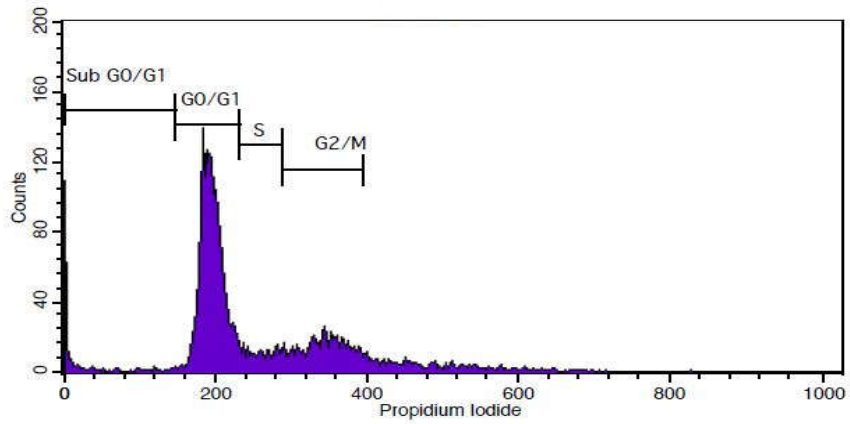


Figure.5.4.1: DNA histogram of HCT116 cells treated with IC_{50} concentration of Indole-3-butyric acid.

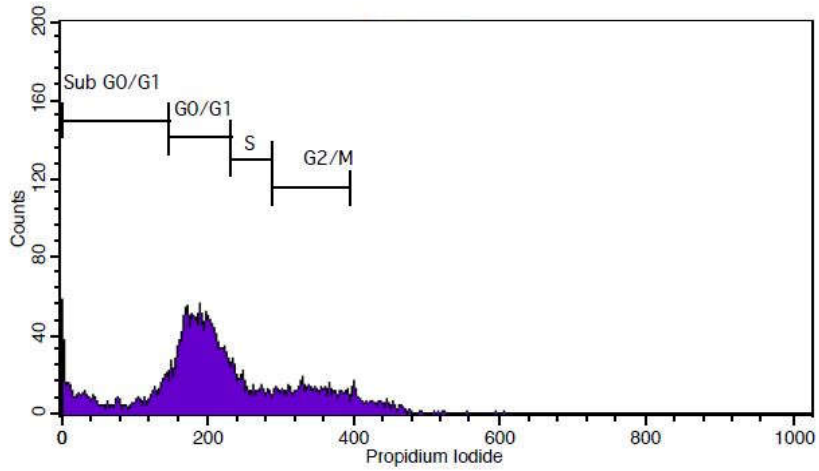


Figure 5.4.2: DNA histogram of HCT116 cells treated with IC_{50} concentration of Tributyrin

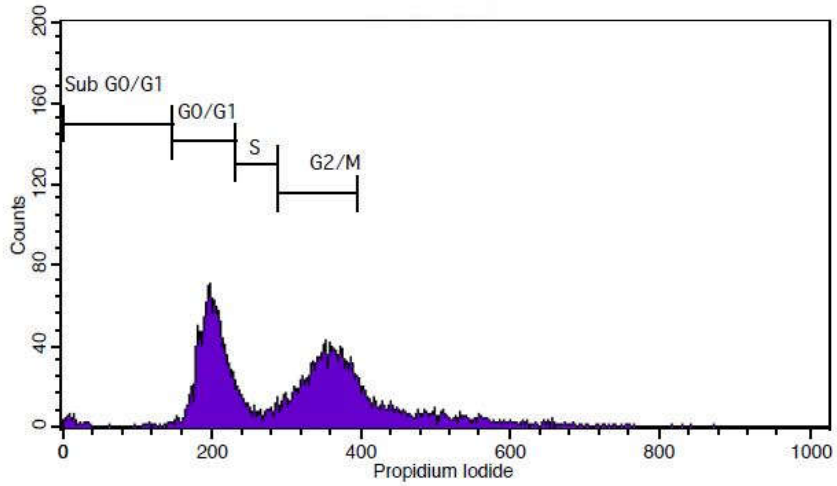


Figure 5.4.3: DNA histogram of HCT116 cells treated with IC_{50} concentration of Standard drug Camptothecin.

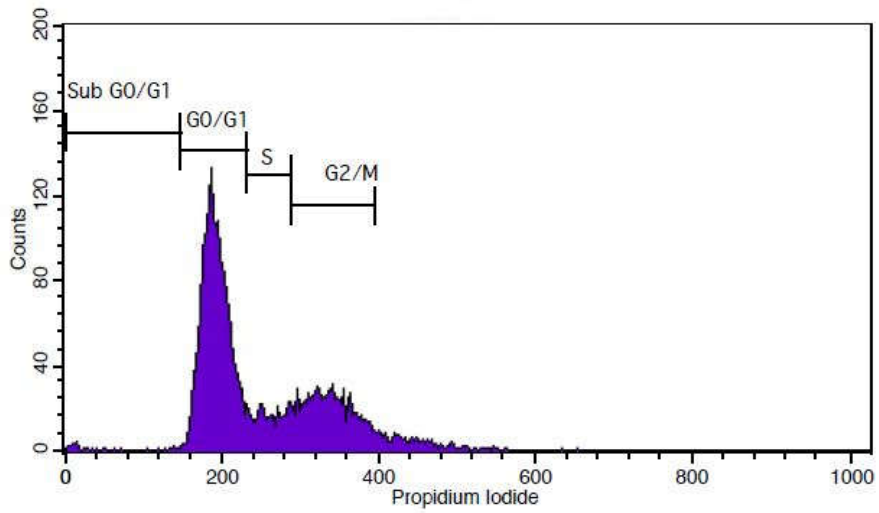


Figure 5.4.4: DNA histogram of untreated control

Table 5.2: Table 4 shows the percentage of cells arrested at sub G_0/G_1, G_0/G_1, S and G_2/M phases

Test compounds	Percentage of Cells arrested			
	Sub G_0/G_1	G_0/G_1	S	G_2/M
Indole-3-butyric acid	4.82 ± 0.1	58.14 ± 0.16	8.17 ± 0.05	20.91 ± 0.86
Tributyrin	16.95 ± 0.07	51.15 ± 1.02	11.18± 0.03	17.07 ± 0.06
Camptothecin	1.29 ± 0.01	36.73 ± 0.13	6.92 ± 0.08	38.63 ± 0.03
Untreated control	0.66 ± 0.02	58.96 ± 0.22	10.93 ± 0.97	25.4 ± 0.14

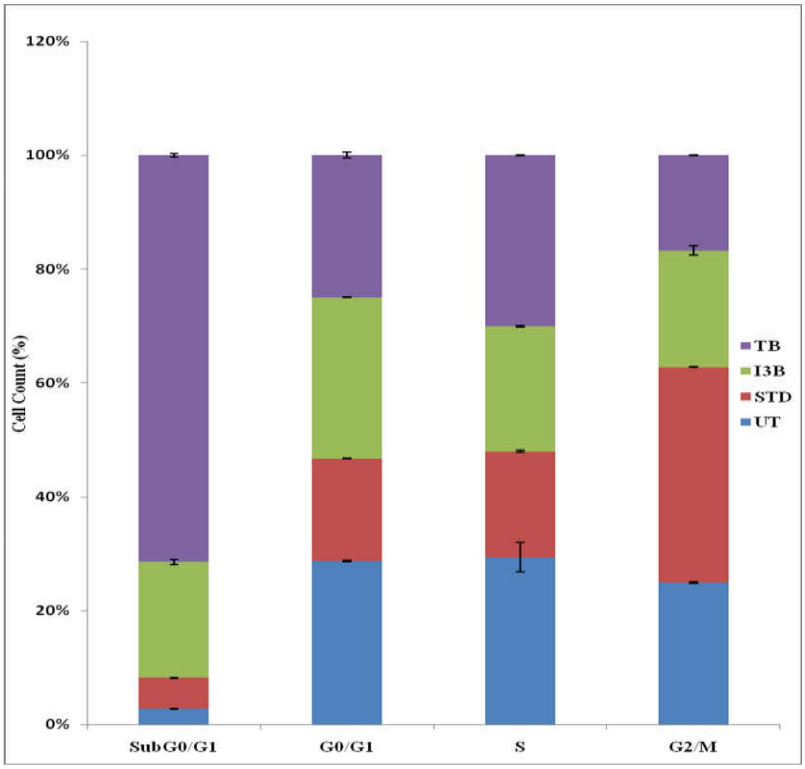


Figure 5.5: Figure represents percentage cell count at cell cycle phases. Analysis in triplicates (significant difference between means at $p<0.05$)

A previous study suggest that the induction of apoptosis and cellcycle arrest by butyrate in bovine kidney epithelial cell lines. It causes cell cycle arrest at G_1/S and G_2/M boundary. The cdc6 and cdk1 proteins downregulated and undergo destruction. Cdc6 regulate the initiation of DNA replication and is required for the prereplication initiation complex in early G_1 phase and cdk2/cyclin A activate the prereplication complex at early apoptosis. The destruction of these complexes leads the DNA damage and hence results in the inhibition of initiation of replication. Cdc6 and cdc2/cdk1 protein complex destruction also causes the activation of caspase-3 and follows

apoptosis (158). The cell undergo DNA damage are activated by ATR (Ataxia-telangiectasia and Rad3-related protein) and ATM (Ataxia-telangiectasia mutated protein). These proteins are the major regulators of DNA damage response. The ATR activates P^{53} and which further activates P^{21}. Cyclin E and Cdk2 (Cyclin dependent kinase 2) are involved in the G_1-S transition. Cyclin E-Cdk2 complex promotes transition into S-Phase. The inhibition of Cyclin E-Cdk2 activity by P^{21} leads G_1 Phase arrest (159).

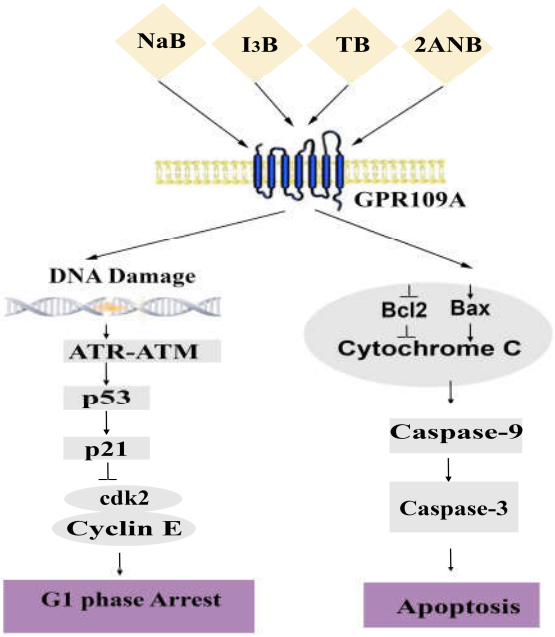


Figure 5.6: Figure illustrates the predicted overall mechanism induced by butyric acid derivatives in HCT116 cells. The DNA damaged cells were activated by ATR-ATM proteins, which activate P^{53} gene and leads P^{21} activation. The inhibition of cdk2-Cyclin E coplex leads the G1 phase arrest. Butyric acid derivatives suppress the Bcl-2 anti-apoptotic protein and enhance the pro-apoptotic protein Bax which leads to the release of cytochrome c from mitochondria which leads Caspase cascade activation and results in apoptosis. NaB: Sodium butyrate, I3B: Indole-3-butyric acid, TB: Tributyrin, 2ANB: 2-Amino-n-butyric acid.

Several studies have shown the cytotoxic effect of butyric acid in different cancer cells (79, 160) and the studies suggest that it induces apoptosis in various types of colon cancer cells (83, 161). Our result clearly show that butyric acid derivatives are the most effective inducer of apoptosis in HCT116 cells. Butyrate induces apoptosis in cancer cells through the activation of caspase-3. Butyrate also induces caspase-3 independent apoptotic cell death in cancerous cells (162).

Sodium butyrate induces autophagy and apoptosis in colorectal cancer cells invitro. Butyrate exert a dose related response in HCT116 cells. At lowest concentration (2 mM) , it induces autophagy. But apoptosis was induces at higher concentration (5 mM) (163). Analogue of butyrate induces direct DNA damage invitro in colon cancer cells and it causes the enhanced proapoptotic effect (164). In this study, we were able to show that the butyric acid derivatives treatment enhances the rate of apoptosis invitro.

Butyric acid is a short chain fatty acid considered as a powerful anti cancer agent for colon cancer cells. The tumour suppressive effect of butyrate in colon is found to be due to the binding of butyrate to the GPR109A receptor (58). GPR109A act as a tumour suppressor protein inducing Short chain fatty acid mediated anti proliferative activity and apoptosis in colon cancer cells. The normal human colon tissue expressed this receptor. The loss of GPR109A (GPR43) expression leads to malignant carcinoma. GPR43 re-expression potentiates the anti-cancer effect of butyrate in human colon cancer (114). In this study, homology modelling and molecular docking were performed to explore structural features and binding mechanisms of butyric acid derivatives with GPR109A receptor.

6.1. Molecular structure prediction

The gene GPR109A code for hydroxycarboxylic acid receptor 2 with 363 amino acid sequnce and 41.85 kDa molecular weight. It acts as a receptor for nicotinic acid as well as (D)-β-hydroxybutyrate and induces greater secretion of adiponectin and lesser lipolysis through the inhibition of adenylyl cyclase. This protein mediates nicotinic acid induced apoptosis in mature neutrophils. Hence, the protein is considered as a potential drug target. The three dimensional structure of target protein was modelled using the best homologous templates by comparative modeling apparoch. The model was visualised by

UCSF Chimera **(Figure 6.1)**. The Ramachandran plot analysis of the modelled protein indicated that 89.0% of residues are present in the most favored region, 8.9 % residues are present in the additional favourable region, 1.8 % residues are present in the generously favourable region and 0.3 % residues are present in the unfavourable region of the plot **(Figure 6.2)**.

The accuracy of a modelled protein is essential for its application in biomedical fields. So the quality of the modelled protein evaluated by developing scoring functions. ProSA plot is a tool that used to predict the absolute quality of protein by evaluating the experimental structures with the statistical significance by comparing its knowledge–based score to random structures with the same sequence. ProSA Z-score is mainly depends on protein size. It is the energy separation between the native fold and random decoy structures, which increases with protein size (165, 166). The hypothetical model was assessed by ProSA and demonstrated the Z-score of -2.11 **(Figure 6.3)**, indicated that the model was in acceptable cut-off range compared with experimental structure. The structural alignment and superimposition performed by Superpose server demonstrated that the three dimensional structure of the target is perfectly superimposed with template structure (human P2Y1 receptor, PDB: 4XNV_A) and the backbone root mean sqaure devaition (RMSD) was estimated to be 0. 73 Å with aligned 532 backbone atoms and 133 α-carbon atoms.

(Figure 6.4). The predicted secondary structure of the model is shown in **Figure 6.5.**

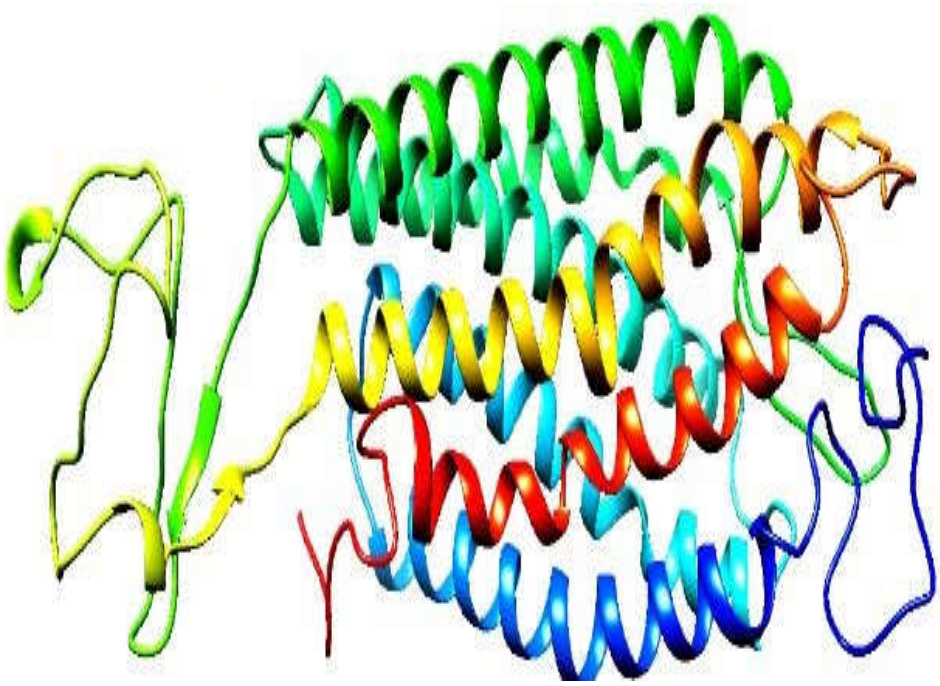


Figure 6.1: The hypothetical model of GPR109A constructed by modeller and visualised using Chimera .

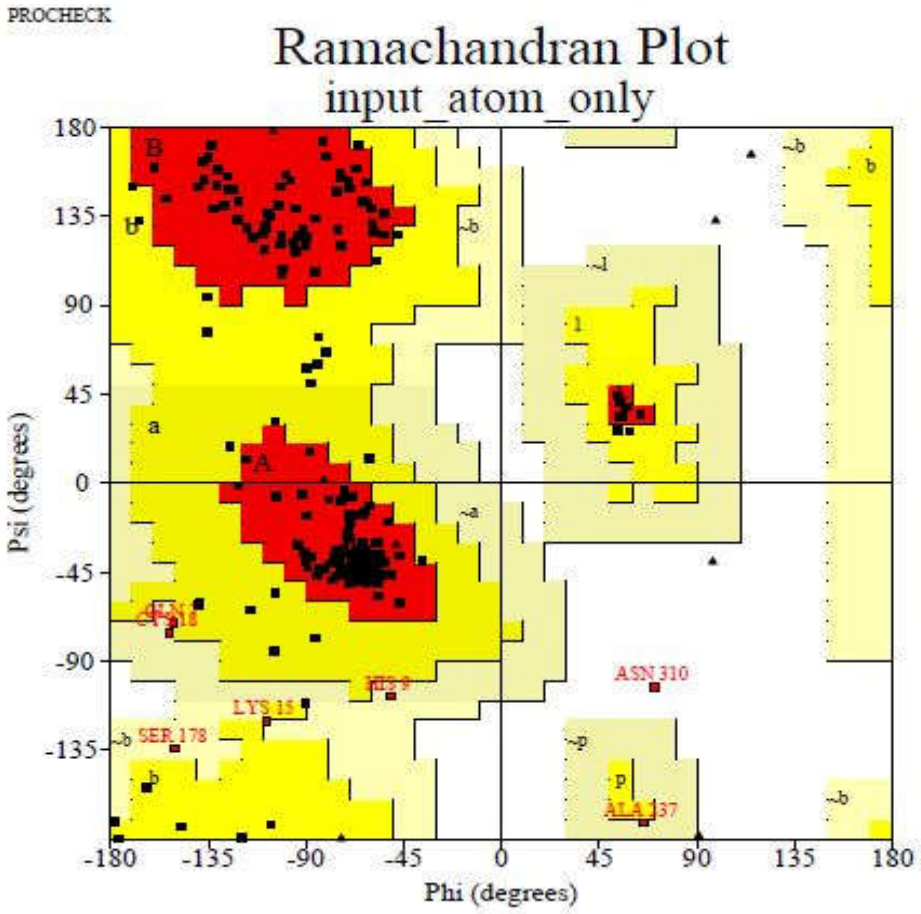


Figure 6.2: Ramachandran plot of the Psi-Phi distribution of modelled GPR109A protein produced by PROCHECK. Residues occupied in most favourable regions : 291 (89.0 %), Residues occupied in additional favourable regions : 29 (8.9%), Residues occupied in generously favourable regions: 6 (1.8%), Residues occupied in unfavourable regions : 1 (0.3%), Number of residues other than glycine and proline residues : 327 (100.0%), Number of end residues (excluding Glycine and Proline) : 1, Number of glycine (described as triangles) : 17, proline residues : 18 and Total residues : 363.

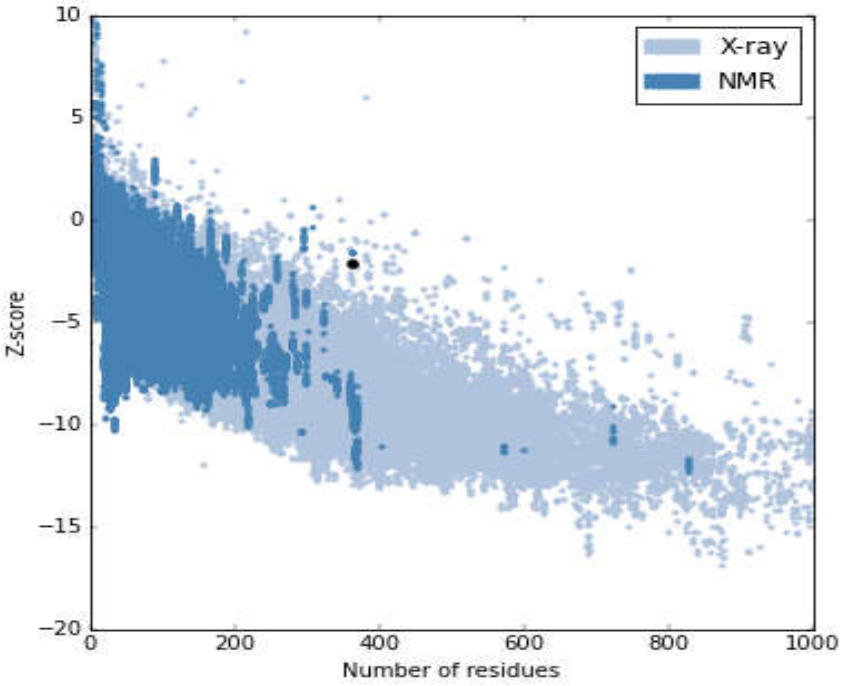


Figure 6.3: Evaluation of the modelled protein structure was done using ProSA plot. The portion with dark blue constitutes the Z-score of protein resolved by NMR and with light blue constitutes the Z-Score of protein resolved by X-ray. The ProSA plot determined Z-Score of -2.11 for GPR109A protein.

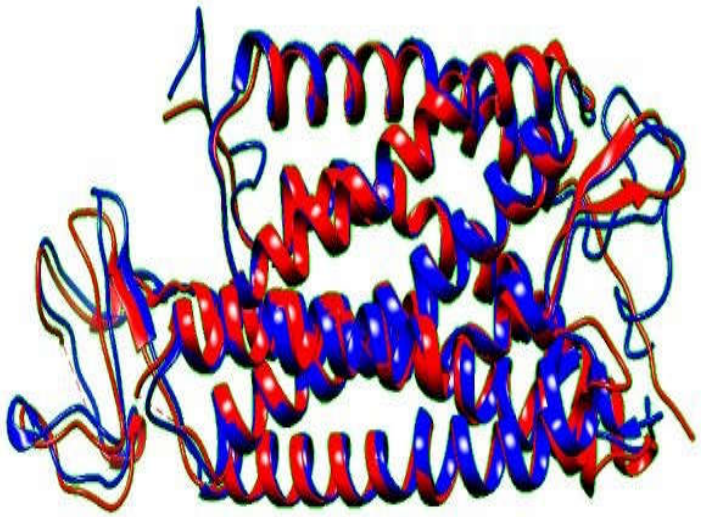


Figure 6.4: The structural alignment and superimposition of the target and template (4XNV, Chain, A). The blue colour represents the target and red colour represents the template in the superimposed image. The backbone root mean square deviation has been identified to be 0.73 Å with 532 superimposed atoms.

Figure 6.5: The secondary structural features of the suspected model was determined by Stride.

The overall quality factor of modelled GPR109A receptor was found to be 48.732 by the tool Errat. The homology modelling studies of GPR109A receptor by Qiaolin Deng et al (2008) suggested that the ligand containing the hydrophobic body as well as the polar tail would enhances the binding affinity (167). Hence the modelled protein possesed most of the crieteria required for considering good quality model.

6.2. Binding potential of butyric acid derivatives against GPR109A receptor using the Molecular docking studies

Flexible body docking study was performed to analyze the binding affinity of the some selected butyric acid derivatives with the putative target, GPR109A. The docked complexes were analysed based on the binding energy, stabilising interactions, cluster RMS, number of hydrogen bonds and interacting residues and docked scores. The affinity of ligands towards the receptor studied by molecular docking is illustrated in **Table 6.1**.

Table 6.1: The best binding pose between GPR109A receptor and butyric acid derivatives performed by Molecular docking

The prioritised target	Ligand	PubChem or Chemspider ID	Binding energy (kcal/mol) of the best docked pose	Amino acids present in the binding cavity
GPR109A receptor	Sodium butyrate	PubChem: 5222465	-4.3	PRO 335
	Indole-3-butyrate	Chemspider:2934315	-6.5	SER 61, PHE 65, VAL 120, VAL 123, ASP 124, PHE 127, THR 143, ILE 147
	Nicotinate	PubChem: 937	-5.8	PHE 301
	Tributyrin	PubChem: 6050	-6.4	ILE 26,LEU 30, LEU 83, TYR 87, TRP 91, TRP 93, LEU 104, ASN 303, ARG 325, THR 332
	2-Amino-n-butyricacid	PubChem: 6657	-4.5	LEU76, LEU107, ARG 111, PRO 335, ARG 339, ASN 336

The molecular docking analysis reveald that the interaction between sodium butyrate and GPR109A is stabilised with the binding energy of -4.3 kcal/mol. Pro 335 is discovered as the main interacting residue in the binding site **(Figure 6.6).** Indole-3-butyric acid interacted with the receptor with the binding

energy of -6.5 kcal/mol. Ser 61, Phe 65, Val 120, Val 123, Asp124, Phe127, Thr143, Ile147 were the residues which are mainly interacting with the ligand **(Figure 6.7).** Similarly, nicotinate interacted to the receptor with the binding energy of -5.8 kcal/mol. Phe301 was discovered as the major interacting residue in the binding site **(Figure 6.8).** Tributyrin exhibited good binding towards the receptor with the binding energy of -6.4 kcal/mol. Ile26, Leu30, Leu83, Tyr87, Trp91, Trp93, Leu104, Asn303, Arg325 and Thr332 were discovered as the major associating residues in the binding pocket **(Figure 6.9).** Further, 2-Amino-n-butyric acid interacted to the receptor with binding energy of -4.5 kcal/mol and Leu76, Leu107, Arg111, Pro335, Arg339 and Asn336 were the important residues present in binding cavity **(Figure 6.10).**

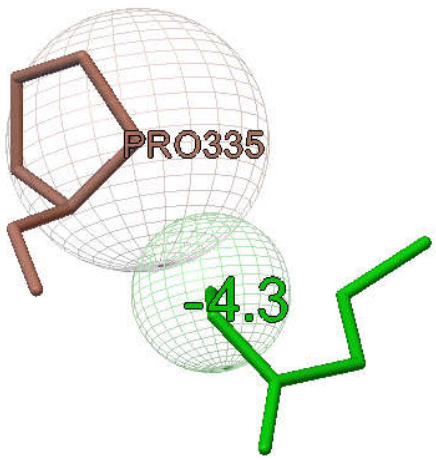


Figure 6.6 : The best binding pose between GPR109A receptor and Sodium butyrate by molecular docking using AutoDock Vina (Note: the green coloured stick is the ligand and surrounding labelled residues are the amino acids present in the binding cavity).

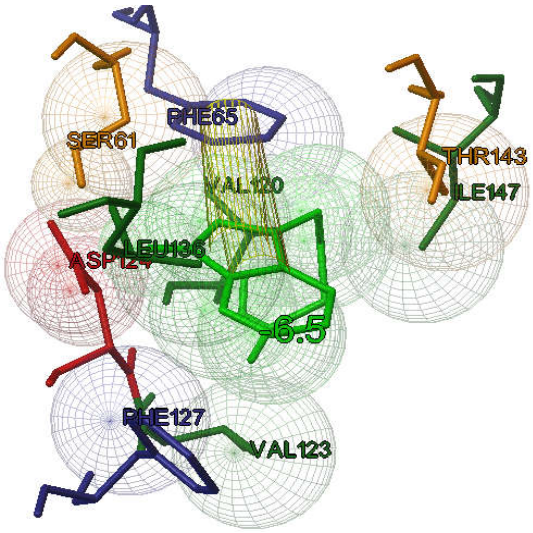


Figure 6.7 : The best binding pose between GPR109A receptor and Indole-3-butyrate performed by molecular docking using AutoDock Vina (Note: the green coloured stick is the ligand and surrounding labelled residues are the amino acids present in the binding cavity). The Pi-Pi Interaction between the aromatics rings of the ligand and one amino acid residue of the receptor

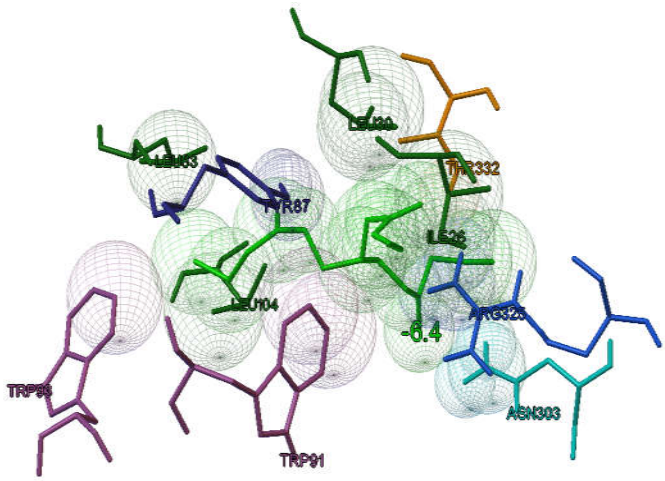


Figure 6.8: The best binding pose between GPR109A receptor and Tributyrin by molecular docking using AutoDock Vina (Note: the green coloured stick is the ligand and surrounding labelled residues are the amino acids present in the binding cavity).

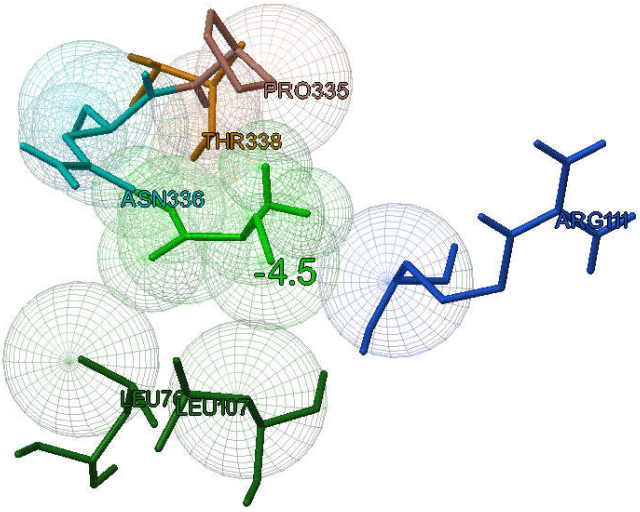


Figure 6.9: The best binding pose between GPR109A receptor and 2-Amino-n-butyricacid performed by molecular docking using AutoDock Vina (Note: the green coloured stick is the ligand and surrounding labelled residues are the amino acids present in the binding cavity)

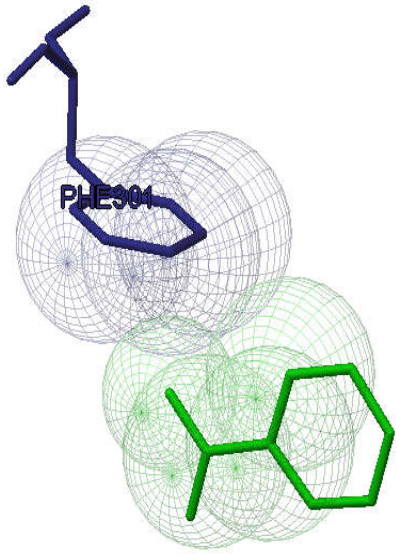


Figure 6.10. The best binding pose between GPR109A receptor and Nicotinate by molecular docking using AutoDock Vina (Note: the green coloured stick is the ligand and surrounding labelled residues are the amino acids present in the binding cavity).

The best docked complexes in the present study was selected based on cluster root mean square (RMS) values, binding energy (kcal/mol), docking score and number of interacting residues especially hydrogen bonds and other weak interaction such as Vander Waal interactions and electrostatic interactions. Although the binding energy of the docked complexes are moderate in the present study, the other parameters such as cluster RMS (which was found to be 0.00), docked score and number of weak interactions were considered for the identification of the best docked poses. Further, studies suggested that the binding energy is not the only criteria for selecting the best binding pose in common docking studies. The studies advised to select the most suitable docking solution as per the scoring function and the additional structural criteria such as number of interacting residues, cluster RMS and docking score of the analogue ligands to ensure the screening of ideal solution for docking (168–171). Thus, the current study used all the described selection criteria to choose the best and ideal docked conformation from the pool of the docking results.

The molecular docking studies suggested that indole-3-butyric acid and tributyrin showed best binding potential against the GPR109A receptor in comparison with other butyric acid derivatives. Re-expression of the receptor depends on the binding energy with the ligands and it is suggested that higher the binding affinity, then more will be the receptor expression. The aromatic ring of indole-3-butyric acid showed pi-pi interaction with GPR109A receptor, which was the direct indication of binding efficiency of these derivatives against the prioritised receptor. Molecular docking is the most widespread method for the modelling of protein-ligand interaction (172). There are

several studies focussed on the binding potential of butyric acid derivatives against GPR109A receptor (173, 174). The receptor is highly expressed in normal human colon tissue, and is silenced sometimes probably due to the promoter methylation followed by transcriptional inactivation (150). The present study suggested that the theoretical binding energy of butyric acid derivatives and GPR109A interaction were comparable with the experimental binding energy between nicotinate, the usual ligand of GPR109A receptor. However, further *in vitro* studies are required to confirm this concept.

The major findings of the study have been summarised as follows;

- After exposure to butyric acid derivatives and nicotinate for 24, 48 and 72 hours, the growth of HCT 116 cells were significantly inhibited (1, 5 and 10 mM for 24 and 48 hours, 1 and 5 mM for 72 hours, p <0.05 and 10 mM for 72 hours, p<0.01). Tributyrin shows highest inhibition rate at 24 hours , 1, 5 and 10 mM concentration.

- The inhibitory effects of butyric acid derivatives on HCT116 cells were suggestive to be dose and time dependent. There was no significant differences between the inhibition rates of tributyrin and 5-fluorouracil for 72 hours at 10 mM concentration.

- The result of combination treatment suggested that at 24 hours, Indole-3-butyric acid and Tributyrin combination shows antagonistic effect and the combinations of Sodium butyrate, 2-Amino-n-butyric acid and Nicotinate with Indole-3-butyric acid and Tributyrin shows synergestic effects. At 48 hours, combination of Indole-3-butyric acid and Tributyrin shows synergestic effect. The combinations of Sodium butyrate and tributyrin (24 hours), indole-3-butyric acid and tributyrin (48 hours), indole-3-butyric acid and nicotinate, sodium butyrate and nicotinate, indole-3-butyric acid and tributyrin and indole-3-butyric acid and 2-amino-n-butyric

acid (72 hours) were maximally induced the cytotoxic effects.

- Among the butyric acid derivatives, Indole-3-butyric acid and Tributyrin shows least IC_{50} value at 24 hours (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 hours (5.84±0.16 and 5.01±0.38 respectively) and 72 hours (4.39±0.25 and 3.30±0.22 respectively).

- The morphological analysis results suggested that the butyric acid derivatives treated cell lines showed low cell confluence, an indicative of apoptosis. Moreover, the nicotinate treated cells are floating in nature, indicated that nicotinate treatment resulted in reduced adherence.

- The result of morphological analysis of cells using acridine orange staining suggested that the cells treated with IC_{50} concentration of butyric acid derivatives as well as nicotinate showed early apoptosis features including membrane blebbing and nuclear fragmentation and this morphological features of apoptosis were comparable with the cells that treated with 5-Fluorouracil, the positive control of the test. Untreated cells showed normal configuration with green nuclei without apoptosis.

- Comets like structure were observed in the cells treated with Indole-3-butyric acid, Tributyrin and Nicotinate under

fluorescent microscope. The result was similar to those found in positive control, 5-Fluorouracil treated cells and the result was an indication of DNA damage.

- The result of Caspase-3 assay shows that treatment of HCT116 cells with butyric acid derivatives as well as Nicotinate significantly activate caspase-3 compared with untreated control. There was no significant difference between the caspase-3 activity of butyric acid derivatives, nicotinate and 5-fluorouracil.

- The result of cell cycle analysis suggested that in Sub G_0/G_1 phase 4.82% and 16.95%, in G_0/G_1 phase 58.14% and 51.15%, in S phase, 8.17% and 11.18 % , in G_2/M phase 20.91% and 17.07% of cell population were present in Indole-3-butyric acid and Tributyrin treated cells respectively. The increase in the Sub G_0/G_1 population indicate the presence of apoptotic cells.

- The three dimensional structure of target protein was modelled using the best homologous templates by comparative modeling apparoch. The Ramachandran plot analysis of the modelled protein indicated that 89.0% of residues were present in the most favored region, 8.9 % residues were present in the additional favourable region, 1.8 % residues are present in the generously favourable region and 0.3 % residues were present in the unfavourable region of the plot.

- The molecular docking studies suggested that indole-3-butyric acid and tributyrin showed best binding potential against the GPR109A receptor in comparison with other butyric acid derivatives. the interaction between sodium butyrate and GPR109A was stabilised with the binding energy of -4.3 kcal/mol. Pro 335 was discovered as the main interacting residue in the binding site. Indole-3-butyric acid interacted with the receptor with the binding energy of -6.5 kcal/mol. Ser 61, Phe 65, Val 120, Val 123, Asp124, Phe127, Thr143, Ile147 were the residues which are mainly interacting with the ligand. Similarly, nicotinate interacted to the receptor with the binding energy of -5.8 kcal/mol. Phe301 was discovered as the major interacting residue in the binding site. Tributyrin exhibited good binding towards the receptor with the binding energy of -6.4 kcal/mol. Ile26, Leu30, Leu83, Tyr87, Trp91, Trp93, Leu104, Asn303, Arg325 and Thr332 were discovered as the major associating residues in the binding pocket**.** Further, 2-Amino-n-butyric acid interacted to the receptor with binding energy of -4.5 kcal/mol and Leu76, Leu107, Arg111, Pro335, Arg339 and Asn336 were the important residues present in binding cavity**.**

Conclusion

The short chain fatty acid butyric acid is synthesized in the colon by the fermentation of dietary fibers in the colon by bacteria and it plays an essential role for preventing colorectal cancer. The present study prioritized GPR109A receptor as a putative drug target as butyric acid mediated apoptosis ws highly depending on the functional perspective of this receptor. The present study has shown that butyric acid derivatives (Sodium butyrate, Indole-3-butyric acid, Tributyrin and 2-Amino-n-butyric acid) as well as Nicotinate mediate Caspase-3 dependent apoptotic pathway in HCT116 cells. The growth inhibitory effects were associated with alteration of cell cycle. Cells were arrested at G_0/G_1 and G_2/M phase.

The computer aided prediction favoured to measure the effective ligands with good binding energy. It was identified that the butyric acid derivatives showed good binding affinity towards GPR109A receptor in comparison with nicotinate, the usual ligand of the receptor. Sodium butyrate, tributyrin, 2-amino-n-butyric acid, indole-3-butyric acid and nicotinate demonstrated growth inhibitory effect on HCT116 cell lines when performed *in vitro* studies. Among the tested derivatives, indole-3-butyrate and tributyrin showed best activity at 10 mM concentration for 72 hours in comparison with other tested derivatives. The same compounds showed the best binding with GPR109A receptor with stabilising interactions and minimum binding energy in computational studies, and the computational prediction was in line with the findings achieved from experimental studies. Tributyrin and

indole-3-butyric acid showed good binding affinity with the GPR109A receptor. Further, these compounds demonstrated profound inhibition rate at 72 hours at 10 mM concentrations in the cytotoxicity assay. Hence, the result is concurrent with computational modelling which suggest that the potential of butyric acid derivatives in colon cancer prevention. Hence, the present study suggested that the butyric acid derivatives probably used to develop therapeutic intervention of colon cancer treatment.

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RPMI-1640 medium

RPMI Liquid media	:	
FBS	:	10% v/v
Antibiotic antimycotic solution	:	1ml/100ml

Cell freezing media

RPMI liquid media		
FBS	:	20 %
Antibiotic-antimycotic solution	:	1ml/100ml
DMSO	:	5 %

Phosphate buffered saline

NaCl	:	137 mM
KCl	:	2.7 mM
Na_2HPO_4	:	8 mM
KH_2PO_4	:	2 mM
P^H	:	7.4

Acridine orange : 5 µg/ml

Ice-cold cell lysis solution

NaCl	:	2.5 M
EDTA	:	100 mM
Tris	:	10 mM
Sodium sarcosinate	:	90 mM
Triton X-100	:	1 %
DMSO	:	10 %
P^H	:	10

Electrophoresis buffer (Comet assay)

NaOH	:	300 mM
EDTA	:	1.2 mM

Neutralisation buffer

Tris-HCl	:	400 mM
P^H	:	7.5

Lysis buffer (Caspase-3 assay)

HEPES	:	50 mM
Triton-X 100	:	5 mM
DTT	:	DTT
P^H	:	7.4

Caspase-3 assay buffer

HEPES	:	20 mM
Triton-X 100	:	0.1 %
DTT	:	5 mM
EDTA	:	2 mM
P^H	:	7.4

LIST OF PUBLICATIONS

1. **Liji Pattayil** and Harikumaran Thampi Balakrishnan Saraswathi. *In Vitro* Evaluation of Apoptotic Induction of Butyric Acid Derivatives in Colorectal Carcinoma Cells: Anticancer Research *39*: 3795–3801, 2019; doi:10.21873/anticanres.13528.

Presentations in Seminars / Conferences

1. **Liji.P**, Sinosh Skariyachan and B.S Harikumaran Thampi; The anti-proliferative activity of butyric acid derivatives in colon cancer cell lines and their molecular docking studies with GPR109A receptor; Abstracts; 85th Annual meeting of SBC(India); 21st -24th November, 2016; 84-85.

2. **Liji.P** and B.S.Harikumaran Thampi; Butyric acid derivatives induces caspase-3 dependent apoptotic pathway in HCT116 cell lines; Abstracts; International conference on neutraceuticals and chronic diseases, Goa, September 1st-3rd , 2017; 96.