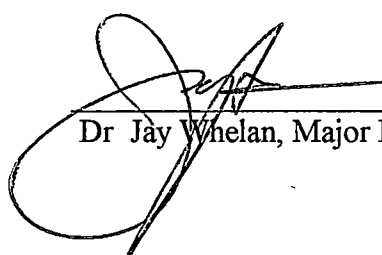


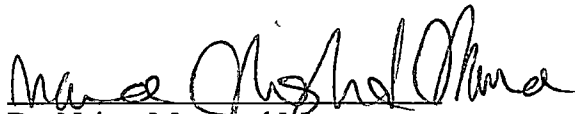
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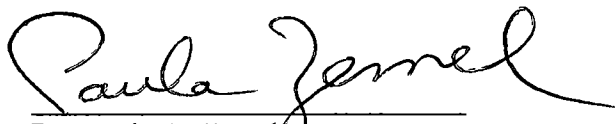
I am submitting herewith a thesis written by Sonia Shahnaz entitled "Effect of butyrate on APC-driven tumorigenesis" I have examined the final copy of this thesis for form and content and recommend that it could be accepted in partial fulfillment of the requirements for the degree of Masters of Science, with a major in Human Ecology



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We have read this thesis
and recommend its acceptance



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Associate Vice Chancellor and
Dean of The Graduate School

**EFFECT OF BUTYRATE ON *APC*-DRIVEN
TUMORIGENESIS**

A Thesis
Presented for the
Master of Science
Degree
The University of Tennessee, Knoxville

Sonia Shahnaz
December 2000

DEDICATION

This thesis is dedicated to my parents Mr Mohammed Ataur Rahman and Mrs. Taslima Rahman of Dhaka, Bangladesh

I am the person I am today as a result of the guidance and direction you have provided. You have shared in my triumph, and helped me through my pitfalls. You have been a source of unwavering love, support, and understanding. I consider myself extremely fortunate to have such a stable foundation on which to build. It would require volumes to express my gratitude for everything you have provided, and my love for you both is beyond measure.

This thesis is also dedicated to my husband, Mr. A. B.M. Mohiuddin, in appreciation for his love, guidance, and invaluable contribution that he has provided me in many facets of life. His continuous support, patience and encouragement enabled me to complete this work.

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ABSTRACT

Butyrate is produced in the colon of mammals as a result of anaerobic fermentation of undigested fiber and has been found to be associated with the maintenance of colonic epithelium homeostasis. Studies have revealed that butyrate plays an important role in decreasing cellular proliferation, promoting differentiation, and inducing apoptosis in a number of colonic adenocarcinoma cell lines *in vitro* and thus provides a basis for investigators to hypothesize that butyrate might have antineoplastic properties against the development of colon cancer. The effects of butyrate on colon carcinogenesis *in vivo* studies still are not clearly delineated. Effects to demonstrate similar effects *in vivo* as observed *in vitro* have failed. This study investigated the role of butyrate in colonic adenocarcinoma cell lines that are defective in the *APC* gene (a tumor suppressor gene that is believed to be associated with the early stages of intestinal tumorigenesis) and the effect of dietary tributyrin (TB, triacylglycerol form of butyrate) on intestinal tumorigenesis in an experimental mouse model with a germ line mutation in the *Apc* gene. *Apc*^{Min/+} mice develop most of their tumors in the small intestines, not in the colon, and thus provide an opportunity to determine whether butyrate is in fact the anti-tumor metabolite of dietary fiber. We demonstrated that butyrate has antiproliferative effects on SW480, SW620 and DLD-1 colonic tumor cell lines. Butyrate inhibited cell growth in a dose-dependent manner with marked reductions of 50-96% at doses between 2.5 and 10mM doses, similar to intracolonic concentrations found *in vivo*. Our *in vivo* study showed that the supplementation of TB to the *Apc*^{Min/+} mice at a level of 6% of energy for 7 weeks failed to reduce tumor load in the colon

or small intestines. It is evident that butyrate may be an important modifier or regulator of tumor cell growth *in vitro*, but its role as an antitumorigenic agent *in vivo* needs further investigation

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LIST OF ABBREVIATIONS

Adenomatous polyposis coli	APC
Azoxymethane	AOM
Cyclooxygenase	COX
Deoxyribonucleic acid	DNA
Dimethylhydrazine	DMH
Ethylnitrosurea	ENU
Familial adenomatous polyposis	FAP
Glycogen synthase kinase-3 β	GSK-3 β
Hereditary non-polyposis colon cancer	HNPCC
Histone deacetylase	HDAC
Inducible nitric oxide synthase	iNOS
Kilodalton	kDa
Messenger ribonucleic acid	mRNA
Millimolar	mM
Modifier of min-1	Mom-1
Multiple intestinal neoplasia	MIN
Nuclear factor- κ B	NF- κ B
Oleic acid	OA
Phospholipase A ₂	PLA ₂

LIST OF ABBREVIATIONS (CONTINUED)

Short chain fatty acids	SCFA
Sodium butyrate	NaB
Tributylin	TB
Trichostatin A	TSA
Tumor necrosis factor	TNF
Weight for Weight	w/w

PART I

INTRODUCTION

Colorectal cancer, next to cancer of lungs, represents the second leading cause of deaths from cancers in the United States (1). It is a common malignancy of Western society and this high incidence is largely associated with dietary factors. Etiological studies strongly indicate that diets rich in fat, meat and animal proteins and low in fiber, fruits and vegetables are causally related to the risk of colon cancer (2,3). Subsequent studies focusing on the role of diet in the prevention of colon cancer demonstrate an inverse correlation between ingestion of a high fiber diet and the risk of colon cancer. It has been reported that poorly fermented fibers (e.g. wheat bran) are protective against colon cancer, whereas the readily fermented fibers (e.g. oat bran) are not protective and may possibly be promotive of tumor development (4-7). The differences observed in the colon cancer incidence with ingestion of different dietary fibers are due at least in part to the fact that the fibers are not a single entity but a group of components with distinct physicochemical properties and thus shows different effects within the colon. Experiments focusing on the role of dietary fiber in colonic homeostasis indicate the importance of fiber-derived fermentation products produced by anaerobic microflora within the lumen of the large intestine (8).

Short chain fatty acids (SCFA) are found to a limited extent in the diet but are primarily produced through the fermentation of the non-digestible carbohydrates by the bacterial flora of the colon (9). Acetate, propionate and butyrate are the major metabolites of anaerobic fermentation of polysaccharides in the large intestine. Of the SCFA produced by bacterial fermentation of dietary fiber, butyrate is of particular importance for several reasons. Butyrate provides the preferred oxidative fuel for normal colonocytes and has a proliferative effect on normal colonic epithelium. In contrast, butyrate has anti-proliferative

and differentiation-inducing effects on a wide variety of neoplastic cell lines in culture when treated with millimolar concentrations of butyrate, similar to the physiological intracolonic luminal concentrations (10-14). Despite the apparent discrepancy between *in vivo* and *in vitro* studies, the anti-growth activity of butyrate on tissue-culture cells has led to the hypothesis that butyrate protects against colon cancer by promoting differentiation, cell-cycle arrest and apoptosis of transformed colonocytes. These antineoplastic roles of butyrate *in vitro* also explain, in part, the apparent protective role of dietary fiber against colon carcinogenesis.

Mutations in the adenomatous polyposis coli (*APC*) gene are thought to be an early event in the initiation of most human colorectal cancers (15). *APC* behaves as a classic tumor suppressor where loss of function results in early benign tumors. For this reason *APC* has been proposed to act as a gatekeeper for colonic epithelial cell proliferation (15). The product of the *APC* tumor suppressor gene has been observed to interact with β -catenin and has thus been proposed to regulate cellular signaling events (16). β -catenin, originally identified on the basis of its association with cadherin adhesion molecules, is now widely recognized as an essential element of the wingless-wnt signaling cascade. In the absence of wnt signals, *APC* simultaneously interacts with the glycogen synthase kinase 3 β (GSK-3 β) and with β -catenin. Phosphorylation of *APC* by GSK-3 β regulates the interaction of *APC* with β -catenin, which in turn may regulate the signaling function of β -catenin. One of the biochemical activities associated with the *APC* protein is down regulation of transcriptional activation mediated by β -catenin and T cell transcription factor-4 (Tcf-4) (17). The protein products of mutant *APC* genes present in colorectal tumors were found to be defective in this activity. In *APC*-

defective cell lines, butyrate has been found to be associated in the induction of apoptosis and it has been reported that butyrate-induced apoptosis increases the formation of the β -catenin-Tcf complex independent of expression of wild-type *APC* and suggests multiple pathways for β -catenin-Tcf signaling (18).

The treatment of mammalian cells *in vitro* with low millimolar concentrations of butyrate causes pleiotropic effects on cellular physiology, including changes in the cell membrane, the cytoskeleton, the cell cycle and gene transcription (19). At least part of the effects induced by butyrate can be attributed to the direct inhibition of the enzyme histone deacetylase (HDAC) (20). The observation is supported by studies using other HDAC inhibitors that mimic the cytological effects of butyrate (20).

However, other mechanisms may be involved to explain butyrate's effect *in vitro*. Butyrate induces cyclin D and cyclin-cdk-inhibitor p21 expression and known to cause growth arrest in G₁ phase of the cell cycle (21). Recently a protein-phosphatase, has been suggested by researchers that could be involved in mediating sodium butyrate's (NaB) effect on deacetylases and constitute an early and essential step in the NaB-triggered signaling pathway (22).

The role of butyrate *in vivo* could not be discussed without discussing dietary fiber. Although the reports of *in vitro* studies have implicated that butyrate might have antitumorigenic properties, the role of butyrate *in vivo* has not been clearly delineated. Subsequent studies with dietary fiber, especially wheat bran, have shown anti-carcinogenic

properties against colon cancer. It is found from most of the studies using carcinogens on rodent models that the distal colon is the primary site of tumor development (23). So it is reasonable to think that if butyrate were protective, then the diets that will produce the greatest concentration of butyrate in distal colon would be more desirable and will show more antitumorigenic properties against colon cancer. But it has been reported from studies that diets containing oat bran produced larger concentrations of SCFA, including butyrate, in both proximal and distal colon, had significantly greater number of tumors than their wheat bran counterparts in a chemically-induced rat tumor model (23).

There is no direct evidence that demonstrates the anti-cancer properties of butyrate in any animal model. Evidence shows that oral doses of NaB enhance the development of colonic tumors in rat model of intestinal neoplasia induced with 1,2- dimethyl hydrazine (24). Another study reported no clinical efficacy of orally administered gastro-resistant pellets of NaB on colon carcinogenesis in the azoxymethane-induced colon tumor rat model. (25). It is possible that the butyrate might get absorbed in the small intestines before it gets into the colon and thus butyrate fails to show the antitumorigenic properties *in vivo* studies, that is why the colon tumor model may be an inappropriate model to evaluate the efficacy of butyrate *in vivo*.

Over the last several years, the $Apc^{Min/+}$ mouse model has been used to evaluate the nutrition intervention on intestinal tumorigenesis because of its germline mutation in the murine *Apc* gene (26). The $Apc^{Min/+}$ mice model is highly susceptible to spontaneous intestinal tumor formation and is currently being used to study human intestinal

tumorigenesis because it genotypically and phenotypically mimics the inherited human disease familial adenomatous polyposis (FAP)(26). Individual with FAP carry a germline mutation in *APC* and mutational alterations or the loss of wild type allele initiates intestinal tumor formation (27). It is reported that somatic mutations that result in loss of full length APC protein occurs early in the spontaneous forms of the disease and indicate an *APC* defect is associated with a majority of colon cancers (28).

This study was conducted to shed further light on the role of butyrate on colon cancer. Although the other forms of butyrate, such as tributyrin (TB), have been found to be more potent than butyrate with respect to inhibition of cell growth and induction of enzyme activity *in vitro* (29), the effect of TB *in vivo* on colon carcinogenesis needs further investigation. In this study, due to a solubility problems of TB in the media (solvent commercially unavailable), NaB has been used to investigate the antiproliferative role of butyrate on a variety of colon adenocarcinoma cell lines in culture that were defective in the APC gene. This study also evaluated the effect of dietary TB on the tumor incidence in *Apc*^{Min/+} mice.

PART II

REVIEW OF THE LITERATURE

Colorectal cancer

The study of colorectal cancer is a fast developing one in the field of cancer epidemiology and preventive oncology. Colorectal cancer has become a major worldwide health problem today. Colorectal cancer, next to cancer of the lungs, represents the second leading cause of deaths from cancers in the United States. Within the United States, all socioeconomic groups have equivalent risk (1).

Colon cancer is judged to be an environmental "man-made" disease based on the incidence patterns around the world. Colorectal cancer is very common in North America, Western Europe, Australia, and New Zealand, whereas the age-standardized incidence rate of colorectal carcinoma is very low in developing countries such as, India and Africa (1,30). Observation on migrants to the United States from Japan, comparing cause-specific mortality rates in 1949 to 1952 with 1959 to 1962, show that the mortality rates of colon cancer among Japanese males have risen in one decade almost equal to the higher risks prevailing for United States Caucasians (31). A similar trend was seen among Polish and South East Asian immigrants to Australia (32). On the basis of variations in the incidence and mortality of colon cancer and increase of risk in populations migrating from low to high risk areas suggest that environmental factors, specifically dietary factors play an important role in the etiology of colon cancer.

The origin of cancer development has been extensively studied during the past twenty years. Both genetic and environmental factors are being considered as risk factors responsible for human colon cancer (33). New ideas about the possible roles of dietary fat,

meat, vegetables, and fiber, and about the metabolic activity of the intestinal bacterial flora and the presence of mutagens in the feces, have opened up lines of research that may reasonably provide the basis for a program of prevention within the next decade.

Molecular genetics of colon cancer

Recently researchers have been able to identify the molecular events that underlie the initiation and progression of human tumors (34,35). The development of human colorectal cancer involves progression through a series of distinct morphological stages and the progressive evolution of colon cancer usually develops slowly over decades. With regard to genetic risk factors, several studies have revealed numbers of activated oncogenes, inactivated tumor suppressor genes and mismatch repair genes that appear to be involved in initiation, promotion, proliferation, and transformation (Table 1) (15,36-39). Abundant clinical and histopathological data suggest that most malignant colorectal tumors arise from preexisting benign tumors (40). Furthermore, both hereditary and environmental factors contribute to the development of colorectal neoplasia, allowing for the study of both inherited and somatic genetic alterations. A model for colorectal tumor development has been proposed that implicates interplay of the accumulated mutations in several tumor suppressor genes and oncogenes in tumor formation and progression (Fig 1) (15,38).

Genetic Alterations during colorectal-tumor development

Activation of oncogenes

Ras gene mutation has been identified as an important type of somatic alteration in colorectal tumors. *Ras* genes encode 21,000-Dalton membrane-bound proteins with intrinsic

Table 1. Genetic risk factors related to colorectal tumorigenesis

Gene	Locus	Length (AA)
Oncogene: <i>K-ras</i> (Kirsten rat sarcoma)	12p12.1	189
Suppressor genes: <i>APC</i> (adenomatous polyposis coli-responsible gene)	5q21	2843
<i>DCC</i> (deleted in colorectal carcinoma)	18q21.3	1447
<i>MCC</i> (mutated in colorectal carcinoma)	5q21	829
<i>p53</i> (protein 53kDa Li-Fraumeni-responsible gene)	17p12	393
DNA mismatch repair genes: (All involved in HNPCC-responsible genes)		
<i>hMSH-2</i>	2p16	1037
<i>hMLH-2</i>	3p21	756
<i>hPMS-1</i>	2q31-33	932
<i>hPMS-2</i>	7q22	862

Source Modified from ref 36

AA: Amino acid, p: short arm of chromosome; q: long arm of chromosome

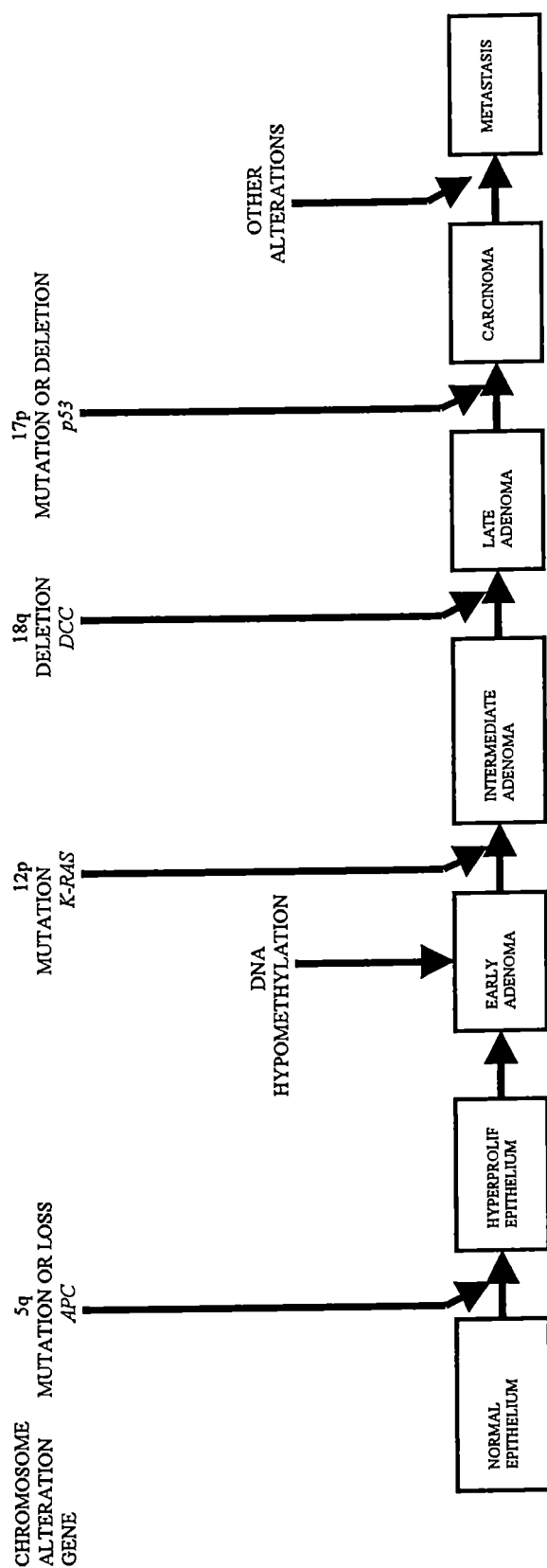


Figure 1. A genetic model of colorectal tumorigenesis

Colorectal tumorigenesis proceeds through a series of genetic alterations involving oncogenes and tumor suppressor genes. Mutation of the *APC* gene leads to abnormal epithelial proliferation and differentiation. An increase in the prevalence of *K-ras* mutation is evident in intermediate adenomas. Deletion of the *DCC* gene occurs more frequently in late adenomas. Deletion and mutation of the *p53* gene is associated with the development of carcinomas (Modified from ref 15, 38)

GTPase activity and presumptive signal transduction capacity. Single base-pair mutations at specific positions within these pairs endow them with the capacity to transform certain cultured cell lines *in vitro*. When transfected into appropriate recipient cells, mutated *ras* gene confer neoplastic properties (34). Approximately 50% of human colorectal carcinomas (41,42) and a similar percentage of adenomas greater than 1 cm in size have been found to have *ras* gene mutations (15).

Loss of tumor suppressor genes

It has been observed that a loss of specific chromosomal regions occurs frequently in colorectal neoplasia. Usually specific regions of one of the two parental chromosomes present in normal cells are lost in neoplasia. These allelic losses put forward the evidence that the regions affected contain tumor suppressor genes and their products normally regulate growth and differentiation and thus indirectly suppress neoplastic development.

Tumor suppressor genes that generally have inhibitory functions to restrain cell growth, proliferation and malignant transformation, were first observed in the 1980s and had drawn the attention of cancer research because they are commonly mutated in human cancer and the spectrum of mutations in these cancers provides clues to the etiology and molecular pathogenesis of neoplasia (15,38).

An inherited predisposition to colorectal tumor formation occurs in an autosomal dominant syndrome, familial adenomatous polyposis (FAP), in which hundreds to thousands of colorectal adenomas develop in affected individuals. Recently, the identifiable locus

associated with this disease has been mapped to chromosome 5q (43,44). Moreover, approximately 20 percent of colorectal cancers from patients without FAP have been shown to have allelic losses of chromosome 5 sequences and it has been hypothesized that the *fap* locus may encode a tumor suppressor gene analogous to that postulated for other inherited tumors (45).

The loss of a large portion of chromosome 17p, has been seen in more than 75 percent of colorectal carcinomas (15,46), but such a loss has been found to be relatively infrequent in adenomas of any stage. Moreover, in several patients the 17p allelic losses were found to be associated with the progression of individual tumors from adenoma to carcinoma. The common region of loss on chromosome 17p in colorectal tumors has been identified and contains the *p53* gene (47). The second most common region of allelic loss in colorectal tumors is chromosome 18q, which is lost in more than 70 percent of carcinomas (15,46) and in almost 50 percent of late adenomas (15). The loss of heterozygosity in chromosome 5q, 17p, and 18q suggest the presence of candidate suppressor genes in particular regions of these chromosomes. Studies directed at the identification of these genes led to the finding of the *p53* gene on chromosome 17p (47,48), the *DCC* gene (deleted in colorectal cancer) on chromosome 18q (49,50) and the *MCC* gene (mutated in colorectal cancer) and *APC* gene (involved in adenomatous polyposis coli), both on chromosome 5q (51-53).

Tumor suppressor genes and recessive models

Tumor suppressor genes have been hypothesized to act "recessively" at the cellular

level, so that both the maternal and paternal copies of the gene must be inactivated in order for the growth suppressive function to be eliminated (54). The predisposition syndromes are generally thought to result from the germline inactivation of one copy of a tumor suppressor gene (a unique gene in each syndrome).

In sporadic tumors, the recessive model predicts that two genetic events are necessary to engender a phenotypic effect. The first event mutationally inactivates one tumor suppressor allele, and the second one inactivates the other (through localized mutation, mitotic recombination, or chromosomal loss).

Multiple allelic losses during tumorigenesis

There are many other chromosome losses besides chromosome 5q, 17p, and 18q in colorectal carcinomas. There are evidences that regions from chromosomes 1q, 4p, 6p, 6q, 8p, 9q and 22q were lost in 25-50% of the cases (49). The allelic loss may reflect two processes First, some chromosomal regions, particularly those on chromosomes 17p and 18q, are lost in the majority of colorectal carcinomas, presumably because they contain suppressor genes that are the "targets" of the losses. Second, many other regions are lost in a more heterogeneous fashion, with each loss often found in only a minority of the tumors. There are two hypotheses to explain these additional allelic losses in individual tumors (49). The first hypothesis states that some of the losses might have no specific effect on the phenotype of the cell, but may have arisen coincidentally with other genetic alterations upon which selection could act, perhaps in complex mitotic events that segregated numerous chromosomes aberrantly Other hypothesis states that there may be many tumor suppressor

genes present throughout the genome, and each of the chromosomal regions lost could contain such a gene. The heterogeneity in allelic loss pattern of individual tumors might therefore be responsible for the observed heterogeneity in the biologic properties of colorectal tumors of different patients (54).

Adenomatous Polyposis Coli (*APC*)

The APC protein is apparently located at the basolateral membrane in colorectal epithelial cells, with expression more pronounced as cells migrate up through the crypt column (55). Expression of wild-type *APC* in colorectal epithelial cells with *APC* mutations results in apoptosis, suggesting that *APC* may control the cell death process (56). Mutations of the *APC* gene are the most common disease-causing genetic events in humans; about 50% of the population will develop colorectal polyps initiated by such mutations during a normal lifespan (57). Individuals who inherit *APC* mutations develop hundreds to thousands of colorectal tumors, consistent with the tumor suppressor or gatekeeping role of APC protein in colorectal tumorigenesis (57). It is one of the early and frequently mutated genes in CRC. Studies directed at the identification of this gene led to the finding of this APC gene on chromosome 5q (51).

APC is a large gene consisting of 15 exons encoding a protein containing 2843 amino acid residues (Fig. 2). Exons 1-14 are small, while exon 15 is large and two-thirds of disease causing mutations are found in the 5' (proximal) segment of this exon (58,59). The *APC* gene product contains heptad repeats at its N-terminus that mediate homo-oligomerization, and seven armadillo repeats of unknown function. The C-terminal part contains a basic

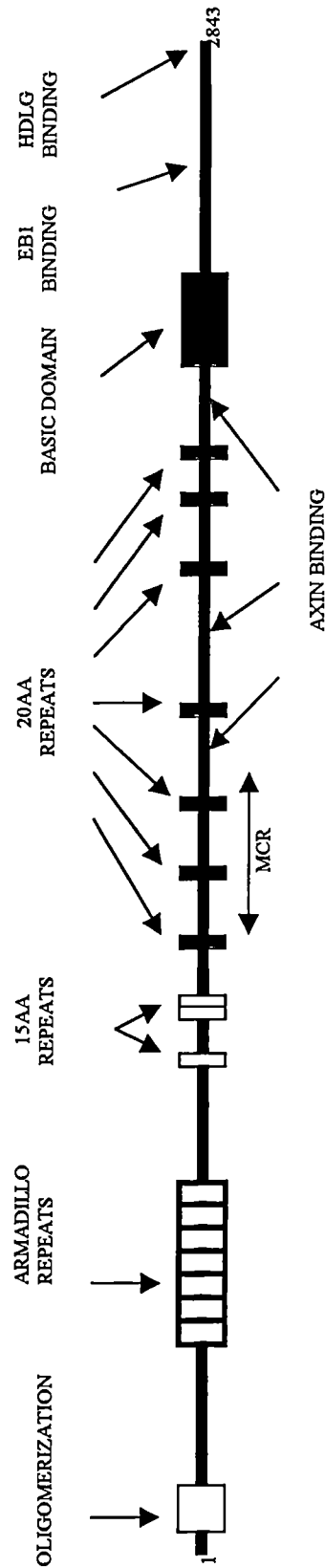


Figure 2. Linear representation of the 2843 amino acid polypeptide chain of the APC tumor suppressor protein

The oligomerization domain mediates homodimerization. The seven armadillo repeats consist of 42 amino acids each. The three repeats of 15 amino acids each independently bind B-catenin and seven repeats containing 20 amino acids each bind B-catenin in a phosphorylation-dependent manner. The basic domain binds microtubules as does the EB1 protein which binds to a carboxy-terminal sequence of APC. Axin binds to three independent sites as indicated. The MCR is the mutation cluster region in which the majority of the APC truncating mutations are localized in sporadic tumors (Modified from ref 60)

domain that binds microtubules, a region interacting with EB1, and a domain that binds the human homolog of the *Drosophila* discs-large protein (DLG). The central part of *APC* contains three 15 amino acid, and seven 20 amino acid repeats that both bind β -catenin. Interspersed within the 20 amino acid repeat region of *APC* are three so called SAMP repeats that have been shown to mediate the interaction with conductin, and by inference, axin (59,60).

The development of intestinal cancer involves progression through a series of distinct morphological stages. Analysis of specific genetic alterations at each of these morphological stages in humans has led to the development of a model implicating accumulation of mutations in several tumor suppressor genes and oncogenes in tumor formation and progression. In FAP patients, somatic mutation of the wild type allele of *APC* is the most frequently detected somatic event associated with tumor formation. In addition somatic mutation of *APC* has been shown to occur frequently in cases of sporadic colon cancer, as well as in hereditary non-polyposis colon cancer (HNPCC), a familial cancer syndrome resulting from inherited defects in DNA mismatch repair (61). These results point to a loss of normal *APC* function as a very early, if not initiating, event in intestinal tumor formation. For this reason *APC* has been proposed to act as a gatekeeper for colonic epithelial cell proliferation (15).

A causal relationship between the loss of *APC* and development of colorectal tumor has been confirmed in mice. Mice heterozygous for an *Apc* gene mutation develop tumors in the intestinal tract following loss of heterozygosity (62). While it remains unclear precisely

how disruption of *APC* function can promote tumor formation, various possible mechanisms have been proposed. One of these hypotheses suggests that, *APC* is a recessive tumor suppressor gene and inactivation of both alleles is necessary for the cancer to develop. This is also known as the “two-hit” hypothesis (63,64). In another hypothesis, chain-terminating mutations in *APC* result in truncated polypeptides, which can inhibit the normal function of the *APC* gene product (61). Other intriguing possibilities have been raised by the recent discovery that *APC* may be an important molecule in regulating cell adhesion (65). This hypothesis is based primarily on evidence that *APC* can bind to and regulate β -catenin, a member of a family of intracellular catenins that regulate cell-adhesion between epithelial cells, in part through interactions with E-cadherin, a family of calcium-dependent homophilic cell adhesion molecules (17,66,67). The β -catenin association links *APC* to two apparently desperate cellular processes (17). The first process is related to cellular adhesion. Several studies have indicated that β -catenin is necessary for cadherin-mediated cell adhesion (65-67). Given that binding of β -catenin to cadherins or to *APC* is mutually exclusive, it is possible that *APC* could modulate such adhesion as part of its gatekeeping function. *APC* could additionally act as a downstream communicator or adhesion status, linking cadherin-catenin complexes to other cellular components.

The second process involving β -catenin has been elucidated via studies of the wntless and wnt signaling pathways in *Drosophila* and mouse respectively (17,65). β -catenin and armadillo (the *Drosophila* homolog of β -catenin) have been firmly implicated as signal transducers in these pathways (65-68). Wnts mainly act on target cells in a paracrine fashion through members of the frizzled receptor family of seven transmembrane spanning proteins

(17) Wnt signaling via the N-terminal cysteine-rich extracellular domain of frizzled leads to activation of the receptors by an unknown mechanism. Activated frizzled receptors induce the stabilization of the cytoplasmic component β -catenin by blocking the function of a multiprotein β -catenin destruction complex (68). This complex consists of the scaffolding component axin, or the related conductin. These bind to the tumor suppressor protein APC, the serine/threonine kinase GSK3 β (glycogen synthase kinase 3 β , zeste-white 3/shaggy in drosophilla) and β -catenin through separate domains. In the absence of a wnt signal, GSK3 β mediates the phosphorylation of β -catenin which is then recognized by the F-box protein slimb/ β TrCP, followed by ubiquitination, and subsequent degradation in proteosomes (17) (Fig.3) In the presence of the wnt protein, the cytoplasmic phosphoprotein dishevelled is activated and interferes with the β -catenin destruction complex. GSK3 β activity is inhibited, and the phosphorylation of the β -catenin is blocked. Hypophosphorylated β -catenin is no longer degraded and accumulates in the cytoplasm and the nucleus where it associates with the transcription factors of the Lef/Tcf family. The Tcf/ β -catenin complexes act as transcriptional activators of wnt target genes and transmit the wnt signal into the nucleus (17,66-68).

Familial adenomatous polyposis (FAP)

FAP is a dominantly inherited autosomal disorder characterized by an early onset of multiple adenomatous polyps of the colon and a high likelihood of colon carcinoma. Patients with FAP typically develop hundreds to thousands of colorectal tumors during their second and third decades of life. Alterations in the human *APC* gene have been demonstrated to play an important role in FAP as well as sporadic cancers. Germline mutations in the *APC* genes

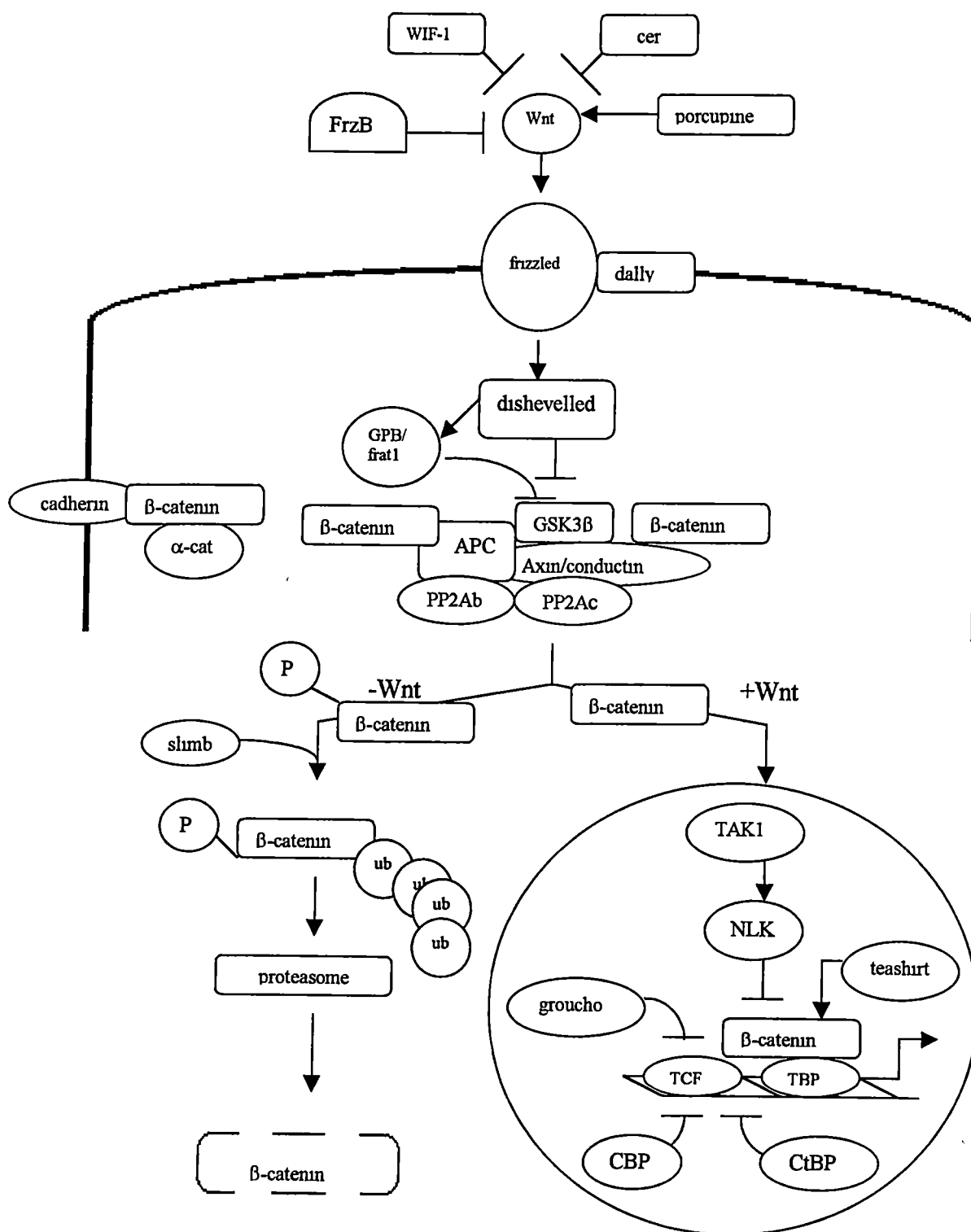


Figure 3. Overview of the wnt pathway
(Modified from ref 17)

are known to be present in individuals with FAP. Somatic *APC* mutations can be found in over 70% of human sporadic colorectal tumors. This mutations occurs as an early event during the colorectal tumorigenesis as frequency of this mutation remains constant when as tumors progress from benign to malignant stages (59). Within last decade, the molecular pathogenesis of FAP has been elucidated where the genetic linkage of FAP and *APC* has been located to a small region of the long arm of chromosome 5 (51,52). Over 200 germline mutations of this very large *APC* gene have been described and most are predicted to result in a truncated protein that is dysfunctional in its role as a tumor suppressor.

Another general principal illustrated by FAP concerns the complex relationship between genotype and phenotype. FAP patients do not develop uniform clinical features despite the fact that all have mutations of the same gene and virtually all mutations result in C-terminal truncated proteins (59). In some cases, the difference in phenotype is due to the type of mutation. For example, retinal lesions (congenital hypertrophy of the retinal pigment epithelium, called CHRPE) are associated with truncating mutations between codons 463 and 1387 of APC gene(69). Truncating mutations N-terminal to codon 157 are associated with an attenuated form of FAP in which patients develop a relatively small number of polyps (70,71). Some studies have suggested that mutations between codons 1250 and 1464 are associated with an increased number of colorectal tumors (61), on the other hand, patients with identical mutations can develop dissimilar clinical features of Gardner's syndrome while others do not develop the syndrome at all (53). It is not known whether these phenotypic differences result from environmental influences or modifying genes.

A compelling example of the relationship between phenotype and APC genotype is observed in *Apc*^{Min/+} mice. These mice develop multiple intestinal adenomas and have a truncating mutation of the murine *Apc* gene at a position similar to that found in many FAP patients (72).

The C57BL/6J-*MIN*/+Mice:

The C57BL/6J-*Min* strain is a congenic derivative of C57BL/6J that is highly susceptible to spontaneous intestinal adenoma formation. *Min* (multiple intestinal neoplasia) is an autosomal dominant trait and involves a nonsense mutation in codon 850 of the murine *Apc* gene. The *Apc* gene is the homolog of human *APC* (adenomatous polyposis coli) gene (72). The C57BL/6J-*Min*/+ mouse presents an opportunity to study pathogenesis of a neoplasia in which the initial molecular defect is similar in humans and in mice.

The *Min* mutation was discovered in the progeny of a C57BL/6J male mouse who was mutagenized by ethylnitrosurea (ENU) in 1990 (75). Sequence analysis of the entire 8535 bp *Apc* cDNA identified that this model, has a fully penetrant dominant phenotype with a germline nonsense mutation (leu (TTG) stop (TAG)) resulting in a stop codon at nucleotide 2549 (codon 850) in the murine homologue of the *APC* gene (*Apc*) (72). This mutation is analogous to those found in human FAP, in sporadic colorectal cancers and in hereditary nonpolyposis colon cancer (71,73,74). The number of intestinal tumors and lifespan in *Apc*^{Min/+} mice are strongly affected by genetic background. For example, (AKR/JxC57BL/6J-*Min*/+) F1 mice develop an average of 5 tumors per mouse and can live beyond 300 days. This change represents a significant decrease in tumor number from the

average of approximately 30 tumors seen in *Apc*^{Min/+} mice derived from C57BL/6J background (26,75). Similar results were found in F1 progeny with two other mouse strains, MA and CAST (75). These findings suggest that the AKR, MA and CAST strains may carry genetic modifiers that act dominantly or semi-dominantly to reduce tumor number in *Apc*^{Min/+} mice. In 1993, linkage analysis demonstrated that a single locus, designated as *Mom-1* (for modifier of Min-1) accounts for much of this difference between strains (76). *Mom-1* is located in a region similar to a segment of human chromosome 1 that was mapped to a 15 cM region on mouse distal chromosome 4. Thus, *Mom-1* seems to play an inhibitory role in the formation of polyps in the *Apc*^{Min/+} mouse. Recently, MacPhee et al. (77) reported that a secretory phospholipase A₂ was an attractive candidate gene for the *Mom-1* locus. This gene *pla2* is mapped to the same region (distal chromosome 4) that contains *Mom-1*, and *Apc*^{Min/+} mouse strains with higher *pla2s* expression develop fewer polyps.

The *Apc*^{Min/+} mouse model is currently being used in cancer research because of close similarities at the genotypic and phenotypic levels with human FAP. This was the first spontaneous murine intestinal tumor model and provides an excellent experimental tool for addressing many fundamental questions associated with intestinal neoplasia.

Dietary factors in human colorectal cancer

Colorectal cancer is a significant cause of mortality in Western societies. Marked differences have been observed internationally in colon cancer incidence, mortality rates, and increase of risk in populations migrating from low to high-risk areas. Evidences suggest that environmental factors, specifically nutritional factors, play an important role in the etiology

of colon cancer (2). The development of colon cancer initially involves one or more genetically altered cells and requires many years to evolve, with a multi step process that affords nutrients an opportunity to modify the evolution of the disease. Compelling experimental and epidemiological evidence indicates that diet and nutrition are key factors in the modulation of colon cancer. Many different dietary factors contact epithelial cells in the colonic crypts both from the luminal contents of the colon and from the basolateral epithelial cell membranes, thereby influencing developmental programs in both normal and transformed colonic epithelial cells. Etiological studies strongly indicate that diets rich in fat, meat and animal proteins and low in fiber, fruits and vegetables are causally related to the risk of colon cancer (2,3). Subsequent studies focussing on the role of diet in the prevention of colon cancer demonstrated that, in populations consuming diets low in total fat, diets high in total fiber, fibrous foods, and certain whole-grain foods reduces risk of colon cancer (2,3,78,79). Numerous studies have shown an inverse correlation between ingestion of a high fiber diet and the risk of colon cancer, thus leading many investigators to hypothesize an antineoplastic role for fiber rich diet.

Dietary fiber and colon cancer

Burkitt first proposed that a diet high in fiber may protect against colon cancer (80). He observed that African Blacks consuming high-fibrous and low-fat foods had lower death rates due to colon cancer compared to their white counterparts consuming low-fiber and high-fat diets. Epidemiologic and animal studies suggest that dietary fiber, especially fiber from cereal sources protects against colon cancer. Subsequent studies on dietary fibers showed that poorly fermented fibers, such as wheat bran, cellulose and lignin, are protective

against colon cancer, whereas the readily fermented fibers, such as oat bran, pectin and guar gum, are not protective and may possibly be promotive of tumor development (4-7). Colonic luminal butyrate concentrations are postulated to be a key protective component of high-fiber diets against colon cancer (23).

The differences observed in the colon cancer incidence with ingestion of different dietary fiber are due, at least in part, to the fact that dietary fibers are not a single entity, but are a complex group of substances with distinct physicochemical properties and thus exert different effects within the colon (23). One of the major differences between dietary fibers is their fermentability, or susceptibility to degradation by the colonic microflora. The fermentation of dietary fiber, and other substrates present in the colonic lumen, results in the production of short chain fatty acids (SCFA), water, CO₂, methane, and H₂ gases (9). In particular, the microbial production of a short chain fatty acid, butyric acid, appears to be important for proper epithelial cell regulation (9).

Postulated protective mechanisms of fiber against colon cancer development

A number of mechanisms have been postulated to interpret the protective role of dietary fiber against the development of colon cancer. It has now been well substantiated that fiber increases fecal volume with resultant dilution of its contents (82). Thus fiber dilutes the primary bile acids, such as, cholic acid and chenodeoxycholic acid and secondary bile acids (deoxycholic acid and lithocholic acid) that are known to be degraded to potential carcinogens by the action of intestinal bacteria (81,82). This dilution process reduces the activity of the bacteria to act on the substrate in the gut. Increased stool volume also dilutes

any carcinogens and co-carcinogens formed in the gut and consequently reduce their carcinogenic activities. Furthermore, the reduced intestinal transit time associated with fiber-rich diets shortens the period of contact between fecal carcinogens and intestinal mucosa and thus confers further protection against colon cancer.

Fermentation of fiber to short chain fatty acid

Short chain fatty acids (SCFA) are found to a limited extent in the diet but are primarily produced through the fermentation of the non-digestible carbohydrates by the bacterial flora of the colon (9). Human colonic bacteria generate a wide range of low molecular weight products in fermentation reactions in the large bowel. Quantitatively, short chain fatty acids are the major end products of both carbohydrate and protein breakdown. These compounds, namely acetate, propionate and butyrate, are the major metabolites of anaerobic fermentation of polysaccharides in the large intestine, but other short chain fatty acids are also produced, including valerate, caproate and branched chain fatty acids isobutyrate, isovalerate and 2-methyl butyrate (82). Lactate and succinate, together with other acidic, neutral and basic substances, are also formed in lesser amounts. Lactate is poorly absorbed by the large intestine so, apart from substrate availability, concentrations in the gut are probably kept low by bacteria that ferment it to SCFA and other products (82).

SCFA are rapidly absorbed from the intestinal lumen. It is assumed that absorption of SCFA in the gastrointestinal tract is passive and that absorption takes place mainly via non-ionic diffusion in the lipid-soluble form (82). However, absorption studies showed that the process is concentration-dependent and that its transport is associated with the

appearance of bicarbonate ions in the colonic lumen, stimulation of sodium absorption and independently of bulk water flux.

Butyrate and the colorectal cancer cell

Of the SCFA produced, the four-carbon monocarboxylic acid butyrate is of particular importance for several reasons. Butyrate has been known to have the capacity to decrease proliferation of a number of colorectal cancer cells *in vitro* (10-13). Butyrate is produced in the colon as a result of fermentation of dietary carbohydrates by anaerobic bacteria. Any butyrate produced is either metabolized by colonocytes or rapidly cleared by the liver, with very little reaching other tissues. Thus butyrate is of interest as a diet-regulated, biologically produced chemical with the potential to suppress the process of tumorigenesis in the colon (14).

Butyrate that is absorbed is efficiently metabolized in the colonic mucosa. It is oxidized completely to CO₂ in the colon, and thus butyrate is the preferred energy source for the colonocytes (83,84).

Butyrate shows growth promoting effects on normal colonic epithelial cells *in vivo* and *in vitro*. In contrast, in a wide variety of malignant cells *in vitro*, butyrate arrests growth and induces differentiation of cultured cells at millimolar concentrations (10-13). Butyrate has been shown in cell culture systems to increase markers of differentiation and induce apoptosis (12,13). These effects suggest that butyrate may be protective of tumor development because it appears to have contrasting effects on proliferation, differentiation,

and apoptosis in normal versus neoplastic tissue.

Phenotypic effects of butyrate

Effects of butyrate on cell proliferation

Butyrate slows cell proliferation and blocks DNA synthesis *in vitro* in a variety of colorectal cancer cell lines including SW480, SW620, HT-29, CaCo-2 (11,13,85). It has been found that at equivalent concentrations butyrate had a much greater influence on the doubling time of SW620 cells than it had on SW480 cell (11). The doubling time of SW 620 cells was increased from 29 hours to 192 hours by butyrate, whereas in SW480 cells the doubling time was only moderately lengthened. Using the human colon adenocarcinoma cell line HT-29, butyrate significantly decreased cell numbers when compared to vehicle control (19). Slowing of proliferation is not permanent, however, and on withdrawal of butyrate the growth rate rapidly increases.

A careful study of the effect of sodium butyrate on DNA synthesis was performed on chick fibroblast and on Hela cells (86). An almost complete arrest of thymidine incorporation was observed in the presence of 2mM butyrate for fibroblasts and 5mM for Hela cells after 24-hour exposure, and this effect has been found to be reversible. After 48 hours of exposure to 5mM butyrate, normal doubling time was observed within 48 hrs following the removal of butyrate. Similarly, thymidine incorporation by HT-29 cells was decreased to 40% of control levels by treatment with 5mM butyrate for 24 hours and cell numbers were reduced to 21% of control levels after 48 hours of treatment (12). This growth inhibitory effect was reversible

Effects on differentiation markers

Brush-border membrane enzymes such as alkaline phosphatase are markers of differentiation, and their expression decreases with neoplastic transformation in the colon (87). Studies with LIM-1215 cultured colon cancer cells incubated with butyrate at 1 mM concentrations demonstrate increased activity of alkaline phosphatase by 600% and increased diaminopeptidylpeptidase by 35% in the LIM1215 cancer cell line (10). Suppression of proliferation and induction of alkaline phosphatase activity occurred at the same time point in culture, suggesting a close link between the differentiating and antiproliferative effect.

Using HT-29 cells, butyrate induces phenotypic changes and enhances alkaline phosphatase activity (19). It was also observed that alkaline phosphatase significantly increased in butyrate-treated cells when compared to control ($p < 0.05$). Others saw a similar increase in alkaline phosphatase activity on butyrate treatment of HT-29 cells (13).

Molecular effects of butyrate

Effects on the cell cycle and oncogenes

Proto-oncogenes in the ras family, *K1-ras*, *Ha-ras* and *N-ras* are believed to be important in growth regulation. These encode GTP-binding proteins, termed p21 (88). In some cells, e.g. NIH 3T3, p21 is needed for the cells to move from G₁ to the S phase of the cell cycle, thus enabling cell division.

A dose dependent effect of butyrate on cell growth has been observed. At

concentrations higher than 4mM, it has been found that 80% of CHO cells were arrested in G₁ phase of the cell cycle, 15% in the S phase and 5% in G₂-M after 24 h (82) Very little effect was observed with 2.5 mM butyrate

Proto-oncogenes in the *ras* family are known to be associated with progression through early stages of adenoma development It has been reported that butyrate causes a dose-dependent reduction in production of p21 in the HT-29 colon cancer cells (88) Expression of the nuclear proto-oncogene *c-myc* is generally associated with cell growth and inversely with differentiation Butyrate reduces *c-myc* mRNA in the CaCo-2 colon cancer cell line by inducing a protein important in its degradation, it also induces *c-fos* mRNA (85) Recently, it has been found that butyrate down regulates expression of *c-myc*, an important growth regulator in LIM1215 colon cancer cells (82) While butyrate decreases the activity of the protein kinase products of the *Src* proto-oncogenes, the significance for this for tumorigenesis is uncertain (89) The effect of butyrate on *p53* has not yet been reported in colon cancer cell lines The effect of butyrate on *APC* or *DCC* also has not been reported Table 2 summarizes the effects of butyrate on molecular events relevant to large bowel neoplasia

Possible molecular mechanism of action of butyrate

Butyrate has been shown to inhibit proliferation and induce apoptosis in a number of human colonic adenocarcinoma cell lines *in vitro* that are defective in the *APC* gene (e.g. SW480, SW620) The effects of *APC* on apoptosis are mediated through its function in modulating β catenin-Tcf signaling The binding of β catenin by APC protein, in a complex

Table 2. Effects of butyrate on molecular events related to colon cancer

Gene	Effects of butyrate on molecular events related to colon cancer	Reference
<i>APC</i>	Unknown	
<i>p53</i>	Unknown	
<i>DCC</i>	Unknown	
<i>c-ras</i>	p21 inhibited	88
<i>c-src</i>	pp60, p56 inhibited	89
<i>c-myc</i>	mRNA reduced	85
<i>c-myb</i>	mRNA reduced	82

Source Modified from reference 82

including axin and glycogen synthase kinase-3 β , targets β -catenin for degradation, thereby generating and maintaining low levels of β -catenin in normal cells (16). It has been found that the loss of wild type *APC* is associated with an increase in monomeric β -catenin. β -catenin forms a complex with Tcf-4, a member of the Tcf-Lef family of transcription factors, which then migrates to the nucleus where it modulates expression of target sequences (90,91). In fact, in *Drosophila*, the proapoptotic effects of mutant *APC* have been demonstrated to be due to up-regulation of the pathway involving signaling through armadillo (the drosophilla homologue of β -catenin) (92), demonstrating the intimate connection between this signaling pathway and regulation of apoptosis. Therefore, it is unclear whether the up or down-regulation of β -catenin-Tcf signaling, associated with mutant or wild type *APC*, respectively, stimulates apoptotic pathways in colonic epithelial cells. Bordonaro et al. demonstrated that unlike wild-type *APC* which down-regulates Tcf activity, butyrate, as well as sulindac and trichostatin A, (an inhibitor of histone deacetylase) induce G₀-G₁ cell cycle arrest and apoptosis in the SW620 colonic carcinoma cell line and up-regulate Tcf activity (18). Moreover, the butyrate-induced increase in Tcf activity, which is reflected in an increase in β -catenin-Tcf complex formation, is apparently independent of the down-regulation caused by expression of wild-type *APC* (18).

The transcription factor nuclear factor- κ B (NF- κ B) has been found to be a widely expressed, inducible transcription factor that plays a central role in regulating the expression of numerous cytokines and cell adhesion molecules involved in immune and inflammatory responses (93). In addition, NF- κ B can play a pivotal role in regulating programmed cell death and has the ability to activate both proapoptotic and antiapoptotic genes (94-96).

Butyrate's effect on colonic inflammatory response may be linked to its influence on NF- κ B activation (97). Most of the NF- κ B in unstimulated cells is regulated by association with one of many inhibitory molecules (I κ Bs). The most widely studied of these is I κ B α that binds to NF- κ B and retains it in the cytoplasm (98). Upon exposure of cells to stimuli, such as, tumor necrosis factor (TNF)- α or interleukin (IL)-1 β , a kinase cascade is initiated which results in activation of the I κ B kinase (99,100). The I κ B kinase phosphorylates I κ B α , which then targets this protein for degradation by the ubiquitin-dependent proteasome (101). Proteolytic degradation of I κ B α allows NF- κ B to enter the nucleus and activate transcription of target genes. The role of NF- κ B in regulating the synthesis of inflammatory mediators in the colon is highlighted by recent findings that NF- κ B activity is increased in inflamed colonic tissue (102). Although most tissues form the p50-p65 NF- κ B heterodimer upon activation, evidence has been obtained that the NF- κ B activated in the colon and small intestine may be primarily a p50 dimer. Exposure of HT-29 cells to butyrate results in a reduction of the constitutive binding of NF- κ B to DNA. Moreover, butyrate is found to selectively inhibit the constitutive p50 dimer as a result of its inhibition of histone deacetylase (97). Evidence for this conclusion comes from the finding that the selective deacetylase inhibitor, TSA, also inhibits p50 DNA binding (97).

Although the molecular mechanisms by which butyrate mediates its effects are not well understood, it is known to induce a variety of changes within the nucleus, including histone hyperacetylation and DNA methylation (103,104). Histone acetylation is correlated with gene activation. The importance of histone acetylation was first proposed by Mirsky and Allfrey who found a correlation between histone acetylation and RNA synthesis (82).

They suggested that the acetylation of histones may affect their capacity to inhibit RNA synthesis. They found that a stimulation of transcription or cell growth is accompanied by an increase in acetate incorporation into histones. It is assumed that acetylated histones are less tightly bound to DNA, decreasing electrostatic bonds (105). The consequence of this could be a release of the bonds between DNA and histones that results in an increase of the accessibility of DNA not only to nucleases but also to various factors involved in the control of gene expression. It is assumed that butyrate treatment alters the rate of chromatin digestion and the rate of transcription since it increases the amount of acetylated histones.

Histone hyperacetylation generally has been associated with both a decrease in cell growth and the activation of specific genes, effects similar to those of butyrate (105). Effects of butyrate attributable to histone hyperacetylation can be assessed by comparing the effects of butyrate to those of TSA, a compound that has been shown to be a very potent and specific inhibitor of histone deacetylase at very low concentrations (20). In a variety of cell types, butyrate and TSA have been shown to cause a G₁ cell cycle arrest. The cyclin-dependent kinase, cdk inhibitor p21 also is known to exert a G₁ cell cycle arrest in response to a variety of stimuli (20).

The treatment with butyrate strongly stimulates cyclin D and p21 expression, while unaffected cdk4, cdk6, and p16 level. Cyclin D/ cdk4 and cdk6 complexes are thought to play a major role in cell cycle progression in early G₁ phase. Cyclin D may directly participate to the inhibition of proliferation (21). Moreover, the expression of transcription factors involved in cell growth, namely *c-myc* and *c-jun* were also reduced by cyclin D1 over

expression (21) Butyrate which blocks the cells in G₁ phase was also shown to reduce the level of expression of *c-myc* in a colonic carcinoma cell line. Cyclins A and E can associate with the cdk2 and have been suggested to control initiation or continuation of DNA replication itself. It has become apparent that cyclin E and A sequentially activate cdk2 around the start of S phase, and the cyclin A activates cdk2 shortly after cyclin E does, concomitant with the onset of measurable DNA synthesis. The diminution of cdk2 level by butyrate may lead to inhibition of the event that is essential for DNA replication (21)

The role of butyrate *in vivo* and tumorigenesis

The protective role of butyrate *in vivo* against tumorigenesis is still unclear. A number of experiments have been conducted to interpret antitumorigenic effect of butyrate *in vivo* by examining the protective role of wheat bran relative to soluble fiber sources. A group of researchers conducted a study to determine if moderate consumption of oat bran or wheat bran fiber could alter tumor incidence in a rodent model of colon cancer, and if the tumor incidence was correlated to concentration of butyrate in the distal colon (23). The result of this study suggested that diets containing wheat bran are protective against colon cancer development. In addition, the experiment showed that large luminal butyrate concentrations in the distal colon alone, as were present in the rats consuming oat bran diets, are not protective of tumor development. Subsequent studies on soluble fibers such as, guar gum or pectin showed failure in elevating distal colonic and fecal butyrate concentrations (5,7).

Summary

There are consistent epidemiological and experimental evidences suggesting the protective effect of dietary fiber against colon cancer. Of the SCFA produced by bacterial fermentation of dietary fiber, butyrate has been suggested as the anti-tumor metabolite of fiber. Although butyrate provides the preferred oxidative fuel for normal colonocytes, neoplastic cell lines have been found to undergo differentiation and apoptosis when treated with millimolar concentrations of butyrate, similar to the physiological intracolonic luminal concentration (10-13). The results of *in vitro* observations with colonic adenocarcinoma cell lines treated with butyrate are quite intriguing and suggest a possible protective role of butyrate in colorectal tumorigenesis and may explain, in part, the apparent protective role of dietary fiber. The report of *in vivo* experiments contradicts what has been shown *in vitro* (24). The effects of butyrate on colon cancer cell lines *in vitro* could not be duplicated *in vivo*. There are other forms of butyrate (e.g. tributyrin) that have been shown to produce growth inhibitory effects in colonic adenocarcinoma cell lines *in vitro* with greater efficacy than sodium butyrate (29). In an effort to test the therapeutic efficacy of tributyrin (TB) *in vivo*, AOM-induced colonic tumor model were treated with TB, but it did not show any clinical impact on carcinogenesis (106). It is possible that TB was digested and absorbed in the small intestines prior to getting to the colon. To evaluate the efficacy of TB on tumorigenesis, colonic tumor models cannot be used as an experimental model *in vivo*. An experimental mouse model that spontaneously develops most of their tumors in the small intestine, *Apc*^{Mim/+} mice has an advantage in the experiments designed to determine whether butyrate is, in fact, the anti-tumor metabolite of dietary fiber.

Research objective

The overall objective of this thesis is to determine if butyrate has anti-tumor properties using *in vitro* and *in vivo* intestinal tumor models with defects in the *APC* gene.

To accomplish this objective, the following specific aims will be investigated:

Specific Aim 1: To determine if sodium butyrate reduces cell growth in a variety of colon adenocarcinoma cell lines with an *APC* mutation.

Specific Aim 2: To determine if the triacylglyceride form of butyrate (tributylin) decreases intestinal tumor load in a murine intestinal tumor model characterized by an *APC* gene defect.

PART III

MATERIALS AND METHODS

Materials

Leibovitz's L-15 culture medium, trypsin/EDTA and fetal bovine serum were obtained from Life Technology (Gaithersburg, MD) RPMI 1640 was obtained from American Type Culture Collection (ATCC, Rockville, MD) Sodium butyrate was obtained from Sigma Chemical (St., Louis, MO). CyQuant Cell Proliferation Assay kits were purchased from Molecular Probes (Eugene, OR)

Cell culture

The human colonic adenocarcinoma cell lines SW480, SW620, and DLD-1 were obtained from the American Type Culture Collection (ATCC, Rockville, MD). SW480 and SW620 cells were grown in Leibovitz's L-15 culture medium, and DLD-1 was grown in RPMI-1640 culture medium. Both media were supplemented with 10% (v/v) heat inactivated fetal bovine serum DLD-1 cells were cultured in Falcon tissue culture flask (70 ml)(Fisher Scientific, Norcross, GA) at 37°C in a humidified atmosphere of 5% CO₂ 95% air, and SW480 and SW620 cells were cultured without supplemental CO₂.

SW480, SW620 and DLD-1 cells were plated in 96 well plates at concentration of 8,000 cells/well, 3,400 cells/well and 14,000 cells/well, respectively, and allowed to adhere. After 24 hrs, the cells were exposed to NaB, dissolved in media, at concentrations ranging from 0.125 mM to 10mM (4 wells for each concentration). The control cells were treated without NaB. At the end of each day, DNA was determined using the CyQuant Cell Proliferation Assay Kit (Molecular Probes, Eugene, OR). Briefly, the media of each plate from each cell line was drained off by turning the plate up side down on a tissue paper. The

plates were covered and stored in -80°C. Cell growth was determined by measuring the amount of DNA/well. The fluorescence measurements were made using a microplate reader (Hewlett Packard, Meriden, CT) with excitation at 485nm and emission detection at 530 nm following the manufacturer's instructions.

Animals

Twenty male C57BL/6J *Apc*^{Min/+} mice (Jackson Laboratories, Bar Harbor, ME), 37 days of age, were randomly assigned to two dietary groups (10 animals /group) on arrival. The mice for the control group were kept in two cages (5 in each cage) and mice for the treatment group were divided into three cages (4, 3 and 3 mice per cage). The animals were not evenly divided in cages because they all were not received on the same day. They were housed in suspended stainless steel cages in a temperature-controlled room with a 12h light/dark cycle and given free access to food and water. The health of the animals were checked daily. Following randomization into dietary groups, all mice were maintained on assigned diets for 47 days. Before sacrifice all animals were fasted overnight. At 85 days of age, they were terminated by cervical dislocation. Each intestinal tract was opened longitudinally and spread out, mucosal surface up, so that the location (large or small intestine), size and number of tumors could be determined using a dissecting microscope (18x). All animal procedures were approved by the University of Tennessee Animal Care and Use Committee and were in accordance with the *NIH Guide for the Care and Use of Laboratory Animals* (National Research Council, 1985).

Diets

The composition of control and treatment diets were based on a typical standard Western diets (Table 3). The macronutrients were 44.4% carbohydrate, 35.4% fat, and 21.2% protein. The base diet was formulated by Monsanto Co. (St. Louis, MO), and Research Diets Inc., (New Brunswick, NJ) with input from Dr. Jay Whelan (University of Tennessee, Knoxville, TN). Tributyrin was added (31 g/kg) to the base diet at the expense of Trisun high oleic-acid (OA, 18:1 (n-9)) sunflower oil (Monsanto Co., St. Louis, MO). Overall fatty acid compositions of the diets are shown in Table 4 and approximates the fatty acid composition of the typical Western diet. All diets were divided into daily aliquots and stored under an atmosphere of nitrogen at -80°C to prevent oxidation. All mice were provided fresh food daily and maintained on assigned diets for approximately 7 wks. Food intake was measured daily and body weight was measured weekly. The food was withheld before the day of killing.

Statistical analysis

The Statistical Analysis System (SAS version 6.12, SAS Institute, Inc., Cary, NC) was employed to analyze the data. Significant difference in the cell culture studies were assessed by ANOVA. Significant differences in body weight and tumor number and size were assessed by t-test at the end of the experiment. Tests for normality and equal variances were performed to check the data. Data in the text and figure are expressed as mean $\pm$ SEM.

Table 3: Composition of the diets

Component	Dietary groups	
	Control	Tributyrin
	Amount (g)	Amount (g)
Casein	22.9	22.9
L-cystine	3	3
Corn starch	274	274
Maltodextrin 10	86	86
Sucrose	114	114
Cellulose	57	57
Cocoa Butter	43	43
Linseed oil	5	5
Palm oil	60	60
Safflower oil	33	33
Sunflower oil, Trisun	31	0
Tributyrin	0	31
Salt mix RD-96	11	11
Di calcium phosphate	15	15
Calcium carbonate	6	6
Potassium citrate	19	19
Vitamin mix V13401	11	11
Choline bitartrate	2	2
Alpha vitamin E acetate	.15	.15
TBHQ	.03	.03

¹The control diet is Monsanto "US 17" diet (Monsanto Co , St Louis, MO, and Research Diets , Inc , New Brunswick, NJ) Abbreviations TBHQ. tertiary butyl hydroquinone.

²Trisun is a high-oleic acid sunflower oil (Monsanto, Inc St Louis, MO).

³Supplied (per kilogram of salt mix) sodium chloride 259g, magnesium oxide 41.9g, MgSO₄ 7H₂O 257.6g, chromium potassium sulfate 1.925g, cupric carbonate 1.05g, sodium fluoride 0.2g, potassium iodide 0.035g, ferric citrate 21.0g, manganous carbonate 12.25g, ammonium molybdate.4H₂O 0.3g, sodium selenite 0.035g, zinc carbonate 5.6g, sucrose 399.105g (Research Diets Inc , New Brunswick, NJ)

⁴Supplied (per kilogram of vitamin mix): all trans retinyl palmitate (500,000 IU per gram) 0.8g, cholecalciferol (100,000 IU per gram) 1.0g, dl-alpha-tocopheryl acetate (500 IU per gram) 10.0g, menadione sodium bisulfite (62.5% menadione) 0.08g, biotin (1.0%) 2.0g, cyanocobalamin (0.1%) 1.0g, folic acid 0.2g, nicotinic acid 3.0g, calcium pantothenate 1.6g, pyridoxine HCL 0.7g, riboflavin 0.6g, thiamin HCL 0.6g, sucrose 978.42g (Research Diets Inc , New Brunswick, NJ)

Table 4: Dietary fatty acid composition of the control diets

Fatty acid	Amount (g/100g total fatty acids)
12:0	0.42
14:0	0.61
16:0	24.44
18:0	12.7
18:1 (n-9)	39.75
18:2 (n-6)	18.98
18:3 (n-3)	2.19
20:0	0.58
22:0	0.32

PART IV

RESULTS

Effect of NaB on cell proliferation

NaB inhibited the growth of SW480, SW620 and DLD-1 colorectal cell lines in a dose-dependent manner. The cells were incubated with NaB for 4 days. It was observed from the study that on day 1 and day 2, the cells started showing responses to NaB with comparatively higher doses of treatment. It is found from the experiments that the growth inhibition pattern was dramatic for all three types of cells on the 4th day. On day 4, SW480 responded to concentrations as low as 0.5mM with marked reductions of 50-95% at doses between 1mM to 10mM (Fig. 4) (Appendix-1). Growth inhibition of SW620 occurred at NaB concentrations starting from 0.125mM. NaB at a dose of 10mM almost completely inhibited the growth of the SW620 cells after 4 days of treatment (Fig. 5) (Appendix-2)). In case of DLD-1, the response to NaB was somewhat mutated compared to the other two cell lines (Fig. 6) (Appendix-3). The growth inhibition was significant at NaB concentrations of 2.5 mM (50% of control) and at 10mM concentration the growth was 16% of the control. NaB did not affect all cell lines equally. NaB had greatest effect on SW620 cells compared to other cell lines.

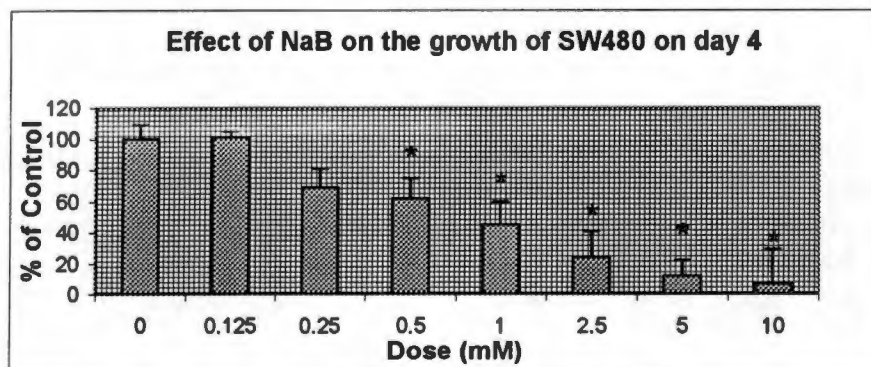
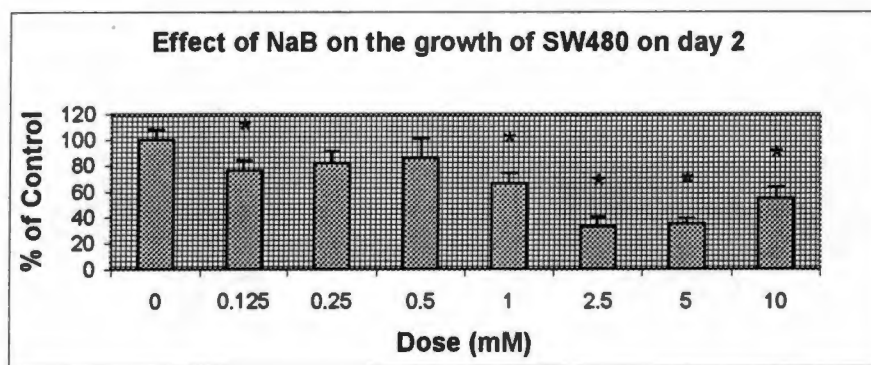
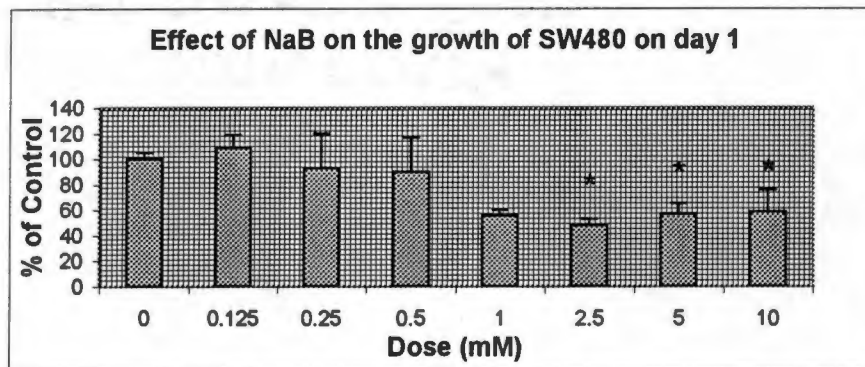


Figure 4. Effect of NaB on SW480 cell proliferation

Cells were treated with increasing concentrations of NaB. Each bar represents the mean $\pm$ SEM (n=4). * Significantly different from the control, $p < 0.05$.

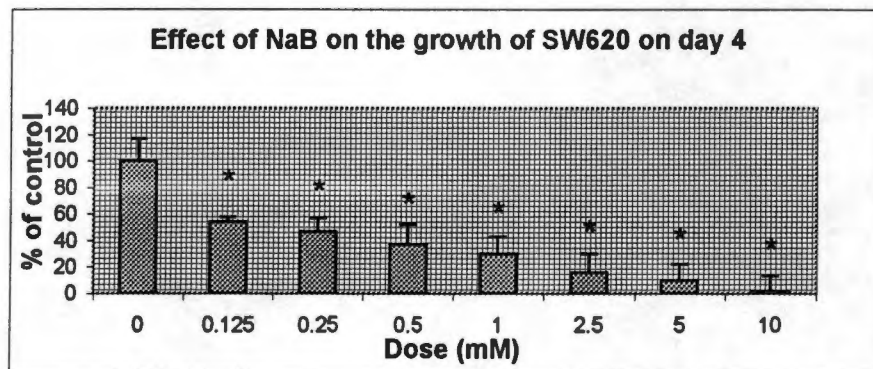
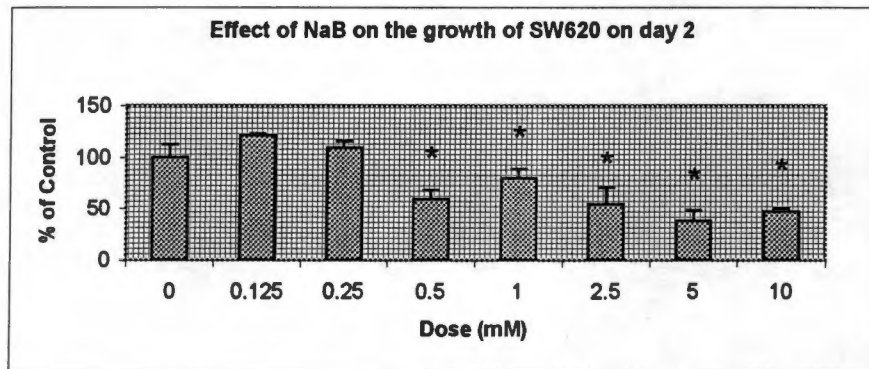
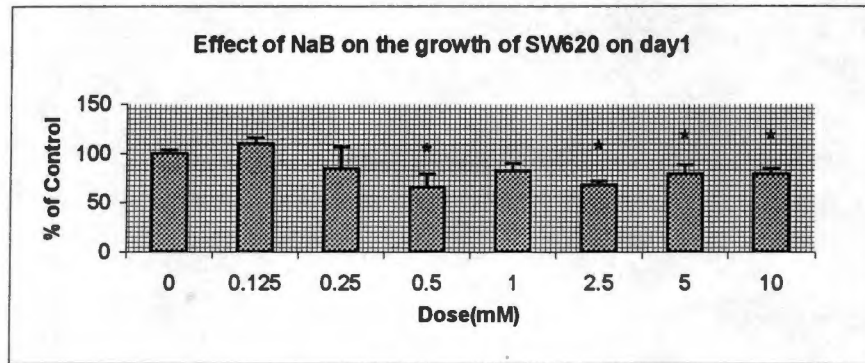


Figure 5. Effect of NaB on SW620 cell proliferation

Cells were treated with increasing concentrations of NaB. Each bar represents the mean $\pm$ SEM (n=4). * Significantly different from the control, $p < 0.05$.

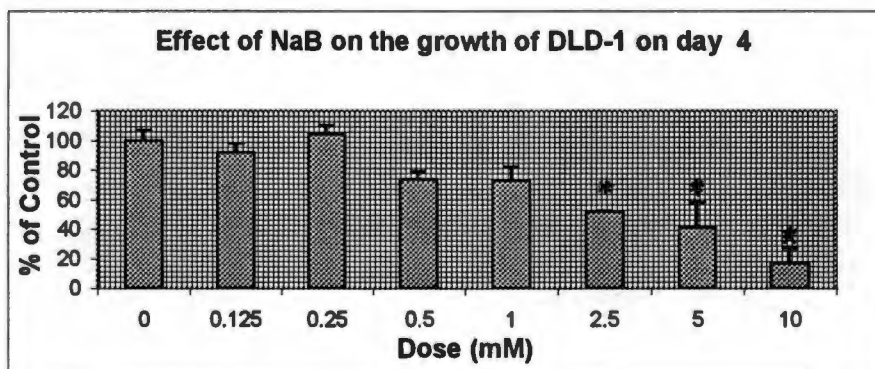
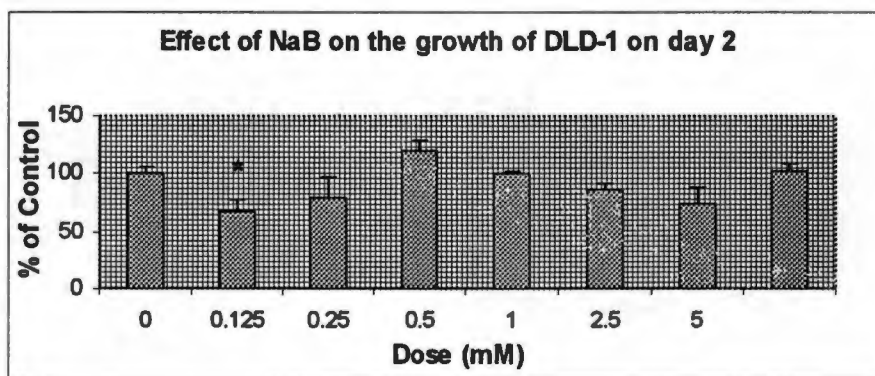
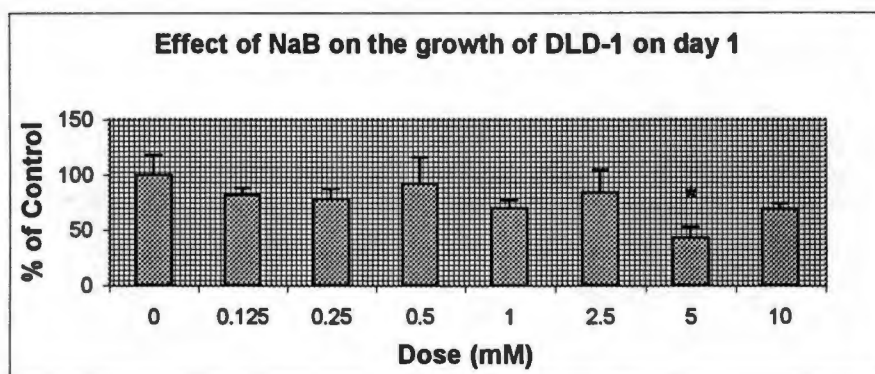


Figure 6. Effect of NaB on DLD-1 cell proliferation

Cells were treated with increasing concentrations of NaB. Each bar represents the mean $\pm$ SEM (n=4). * Significantly different from the control, $p < 0.05$.

Effect of tributyrin on body weight and food intake

There is a significant difference in body weight between the treatment and the control group of mice at week 8 ($t=2.49$; $df=9$; $p=0.01$, one-tailed). Although the two groups started with similar weights (19.9 ± 0.4 g/mouse versus 20.46 ± 0.5 g/mouse), at the end of the experiment the average body weight of the control group was significantly different from the experimental group (32.0 ± 0.8 versus 28.8 ± 0.9) (Fig. 7). Although the experimental group consumed more food than the control group at week 1, it was found from the food intake data that the experimental group started to consume less food compared to the control group through out the experimental period (Fig. 8). The lesser food intake and body weight gain of the mice in the experimental group was not indicative of the increasing tumor load in this case.

Effect of tributyrin on tumor number and size

At 85 days of age, *Apc^{Mim/+}* mice maintained on the control diet had 53.5 ± 7.1 tumors per animal with a mean tumor size of 1.12 ± 0.05 mm (Table 5). The mice in the treatment group had a lower number of tumors than the control group (42.5 ± 4.7 tumor/mice), but this difference was not statistically significant ($t=1.40$; $df=9$; $p=0.09$, one-tailed). The tumor size was unaffected by the treatment.

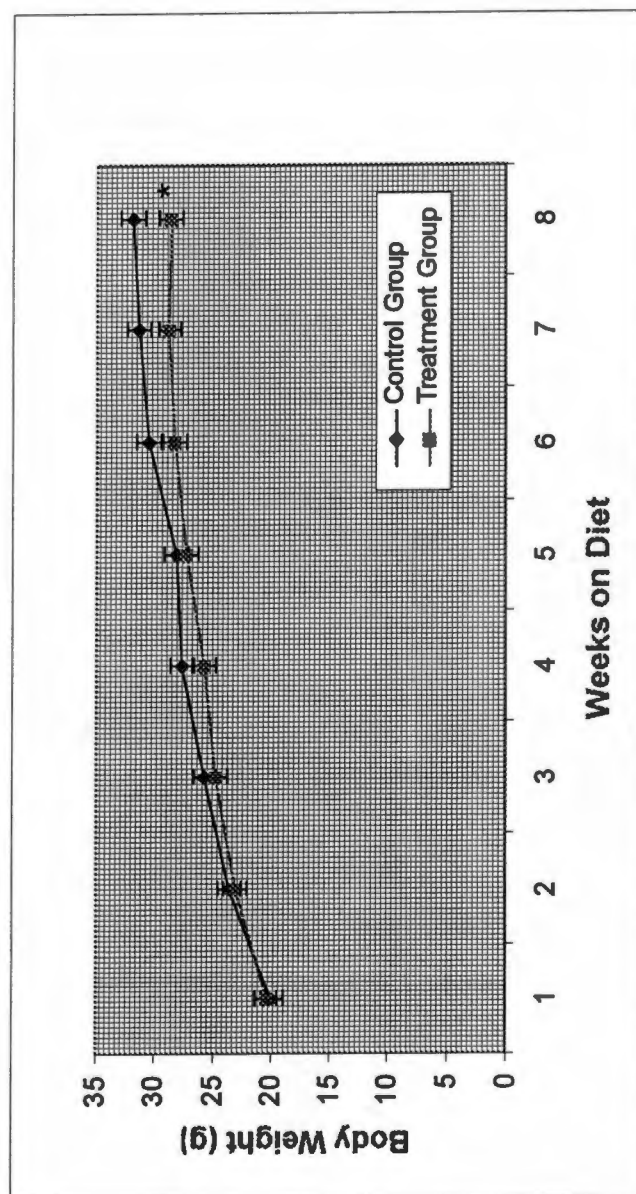


Figure 7. Body weight of *Apc^{Min/+}* mice fed control diets or diets supplemented with tributyrin for 8 Weeks

Data are means $\pm$ SEM, ($n=10$). Final weights were evaluated for significance by t-test.

* Represents significant difference from the control at $p < 0.05$.

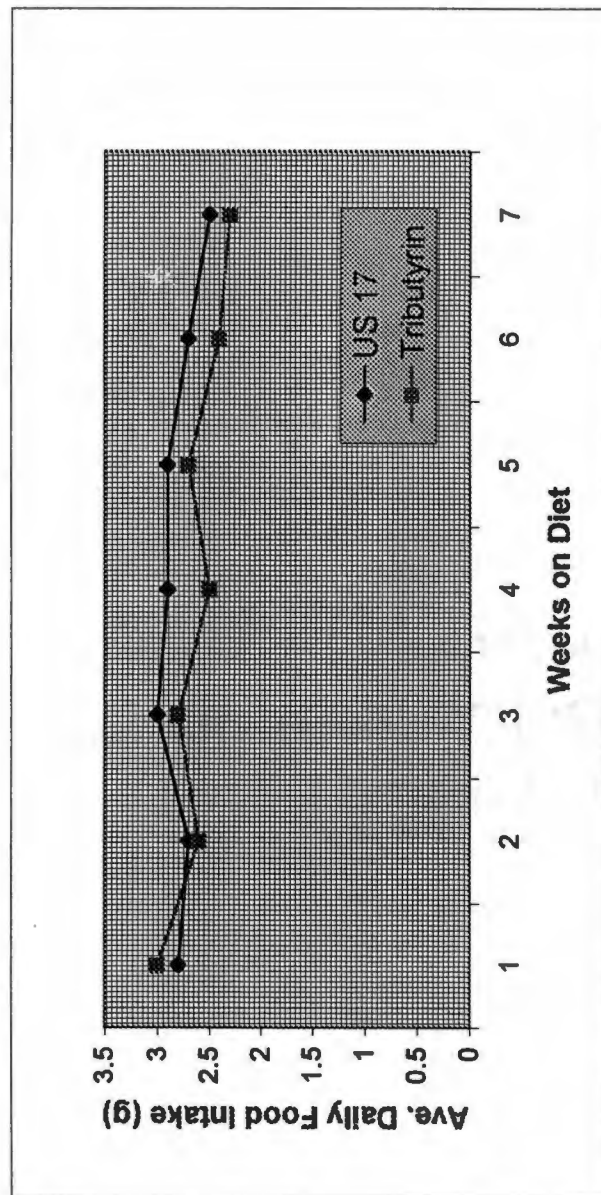


Figure 8. Food intake of *Apc^{Min/+}* mice fed control diets or diets supplemented with tributyrin for 8 Weeks

Table 5. Effects of Tributyrin Treatment on Tumor Size and Number in the Colon and Small Intestines of *Apc*^{Mm/+} Mice

Tumor Size	Dietary Group ^a	
	Control (n=10) Total Tumor /Number/Group	Treatment (n=10) Total Tumor Number/Group
Colon (mm)		
<1	- ^b	- ^b
1-1.5	2 ^c	1
1.75-2.25	1	1
2.5-3.0	2	2
3.25-4.0	6	2
Small Intestine (mm)		
<1	153	114
1-1.5	286	220
1.75-2.25	75	52
2.5-3.0	10	7
3.25-4.0	- ^b	3
Total #/ Group	535	425
Ave. Tumor/Mouse ^d	53.5±7.1	42.5±4.7
Ave. Tumor/ size ^e	1.12±0.05	1.13±0.05

^a *Apc*^{Mm/+} mice in the control group were treated with US17 diet and *Apc*^{Mm/+} mice in the treatment group were supplemented with 3% (w/w) tributyrin diet for 48 days. Tumor number and size were examined from the entire intestinal tract using a dissecting microscope (18x).

^b None observed

^c Number of tumors

^d Mean ±SEM (n=10 animals/group)

^e Average size (mm) was calculated as a weighted average for each size classification and expressed as Mean±SEM.

PART V

DISCUSSION

Colorectal cancer is a common malignancy of Western society and this high incidence is thought to be largely associated with dietary factors. Burkitt originally proposed that a diet high in fiber may protect against colon cancer (80). Studies on dietary fiber suggest that poorly fermented fibers, such as wheat bran, cellulose and lignin, are protective against colon cancer, whereas, the readily fermentable fibers, such as oat bran, pectin and guar gum, are not protective and may possibly be promotive of tumor development (4-7) Butyrate is produced in the colon of mammals as a result of microbial fermentation of dietary fiber, undigested starch, and proteins (9) Butyrate is of particular importance for several reasons (23) First, butyrate is the primary energy source for the colonocytes; it is utilized preferentially over glucose, glutamine, and the other short chain fatty acids Second, butyrate has been shown to increase cellular proliferation in normal colonocytes, a property that has been suggested as a risk factor for colon tumor development. Finally, butyrate has been shown in cell culture systems to increase markers of differentiation and induce apoptosis. These effects suggest that butyrate may be protective of tumor development because it appears to have contrasting effects on proliferation, differentiation and apoptosis in normal versus neoplastic tissues. Although a number of studies demonstrate an inverse correlation between mortality rates from colon cancer and dietary fiber consumption, the results of two recently published studies have raised questions about the efficacy of dietary fiber in reducing the risk of colon cancer (107,108) It has been reported that a daily wheat-bran supplement (for 3-4 years), or following a diet that was low in fat and high in fruits and vegetables had no effect on the incidence of new colorectal adenomas It was reported in study by Alberts et al and Shatzkin et al.,(polyp prevention study) that the incidence of

cancer was slightly higher in the intervention groups than in the control group (7 cases vs. 2 and 10 cases vs. 4 respectively) These differences among studies conducted with dietary fibers have generated much debate regarding the role of butyrate in the colon and colorectal tumorigenesis. In our study, we evaluated the antineoplastic role of butyrate *in vitro* with cell lines that were defective in the *APC* gene and the effect of butyrate on intestinal tumorigenesis *in vivo* in an experimental mouse model with a germline mutation in the murine *Apc* gene.

Mutations in the *APC* gene are thought to be an early event in the initiation of most human colorectal cancers (15). *APC* behaves as a classic tumor suppressor where loss of function results in early benign tumors. For this reason *APC* has been proposed to act as a gatekeeper for colonic epithelial cell proliferation (15). Our *in vitro* studies have focused on the antiproliferative role of butyrate on colon adenocarcinoma cell lines that are defective in *APC*. Butyrate inhibited the growth of different colorectal cell lines in a dose-dependent manner and similar results have been reported by others (10-13,85) At concentrations of less than 10mM, sodium butyrate produces a number of morphological and biochemical modifications in cultured cells resulting in the inhibition of proliferation and augmentation of apoptosis. These results are observed at concentrations far lower than the 20mM found in the large intestines of humans following a high fiber diet and lower than the concentrations ($\geq 10\text{mM}$) that produce cytotoxic effects *in vitro*.

Our observations showed that NaB did not affect all cell lines equally. NaB had the

greatest effect on SW620 cells, as compared to SW480 and DLD-1 cells, which is in accordance with the findings of Kim et al. (11). At the same concentrations used in our study, butyrate had a much greater influence on the doubling time of SW620 cells than SW480 cells and the cells appeared flatter and larger and displayed long membranous processes compared to the untreated cells (11). We did not observe any apparent morphological changes with any cell type, but we only exposed our cells to butyrate for 4 days as compared the 8 day treatment by Kim et al.

Although butyrate provides the preferred oxidative fuel for normal colonocytes, neoplastic cell lines have been found to undergo differentiation and/or apoptosis when treated with millimolar concentrations of butyrate. Treatment of mammalian cells *in vitro* with millimolar concentrations of butyrate has pleiotropic effects on cellular physiology, including changes in the cell membrane, cytoskeleton, the cell cycle and gene transcription. It was observed in HT-29 cells that butyrate-induced cell differentiation is associated with increased activity of alkaline phosphatase and changes in the lipid composition (19). Differentiated cells showed increased level of triacylglycerol, cholesteryl esters, and decreased free cholesterol and phospholipids (19). Furthermore, colonic tumor cell lines, including premalignant adenoma lines, undergo apoptosis in response to physiological concentrations of butyrate and concentrations needed to induce apoptosis were similar to the concentrations that caused hyperacetylation of core histones in the VACO-5 cell line (109).

Other forms of butyrate have also been tested with great success. With respect to

inhibition of cell growth and induction of enzyme activity, tributyrin (TB) is about 2 fold more active than butyrate (29). On the assumption that complete hydrolysis of 1 mol TB by lipases would yield 3 mols of butyric acid one would expect a 3 fold higher potency for TB on a molar basis. It is evident from the study of Schroder et al. that treatment with 1mM TB on HT-29 cells for 6 days caused a growth reduction approximately to that observed with 2mM NaB (29). The effects of TB may be attributed to a faster rate of penetration through cellular membranes and to a lower rate of metabolic degradation as a result of higher intracellular butyrate concentration. For our *in vitro* study, we used NaB (instead of TB) due to its high solubility. It has been reported by others that TB requires emulsification with Lipofundin MCT 10% (commercially unavailable) to minimize solubility problems in the culture medium.

The mechanisms explaining butyrate's effects *in vitro* remain uncertain and potentially are multifactorial. The cell lines and murine model used in these studies have associated defects in the *APC* gene resulting in enhanced β -catenin-Tcf signaling. It is believed that nuclear localization of the β -catenin-Tcf complex regulate downstream events, such as proliferation and apoptosis. However, it has been reported that butyrate-induced apoptosis increases the formation of the β -catenin-TcF complex in SW620 cells independent of expression of wild type *APC* (18), suggesting multiple pathways for β -catenin-Tcf signaling (110). These same authors report that sulindac (a non-selective cyclooxygenase inhibitor), like butyrate, also increases Tcf signaling. These results are in apparent conflict with *in vivo* data demonstrating a down-regulation of β -catenin levels in tumors of sulindac-

treated *Apc*^{Mim/+} mice (111).

Although the molecular mechanisms by which butyrate mediates its effects are not well understood, butyrate is known to induce a variety of changes within the nucleus, including histone hyperacetylation and DNA methylation (104,105). Histone hyperacetylation generally has been associated with both a decrease in cell growth and the activation of specific genes, effects similar to those of butyrate (105). In a variety of cell types, butyrate and the histone deacetylase inhibitor trichostatin A (TSA) have been shown to cause a G₁ cell cycle arrest. These effects could be related to butyrate's influence on NF- κ B activation and histone acetylation (97). NF- κ B exists in the cytoplasm as homo or heterodimers. Exposure of HT-29 cells to butyrate results in a reduction of constitutive NF- κ B DNA binding due most likely to a reduction in NF- κ B nuclear localization. Butyrate was found to selectively inhibit p50 dimer nuclear localization without inhibiting expression and since TSA-treated cells replicated butyrate's effects, the ability of butyrate to inhibit histone deacetylase may be related to its effects on NF- κ B signaling (97).

Other mechanisms may be involved, however. Treatment with butyrate strongly stimulates cyclin D and cyclin-cdk inhibitor p21 expression, and can induce G₁ cell cycle arrest (20,21). Treatment of cells with NaB is known to increase histone acetylation by inhibiting deacetylases. It has been observed that in cultured hepatoma cells the potent serine-threonine phosphatase inhibitors okadaic acid or calyculin A inhibited phosphatase activity and concomitantly decreased the histone acetylation maintained by NaB (22). These

results suggest that a protein phosphatase may mediate NaB's effect on deacetylases and may constitute an early and essential step in the NaB-triggered signaling pathway.

Although the reports of *in vitro* studies have implicated that butyrate might have antitumorigenic properties, the role of butyrate *in vivo* has not yet been clearly delineated. There is no direct evidence that demonstrates the anticancer properties of butyrate in any animal model. On the contrary, one paper reports that oral doses of NaB enhances the development of colonic tumors in a rat model of intestinal neoplasia induced with 1,2-dimethylhydrazine (24). In addition no clinical efficacy was observed following orally administered gastro-resistant pellets of NaB on colon tumorigenesis in the azoxymethane (AOM)-induced colon tumor rat model (25). In our study we treated *Apc*^{Min/+} mice with TB to evaluate its effect on intestinal tumorigenesis. Our study revealed that TB has no clinical impact on intestinal tumorigenesis even though most of the tumors are localized in the small intestine. Our data showed no significant changes in the tumor number and size after the mice were fed a diet containing TB at a level of 6% of energy for 7 weeks. In a similar experiment using AOM-induced colonic tumor rat model Deschner et al. reported that feeding tributyrin for 6 months increased fecal butyrate levels 10 fold, but no differences in AOM-induced focal areas of dysplasia or colonic tumor incidence was observed between tributyrin fed and control animals (106). In the human trials, it is unlikely that plasma concentrations of butyrate would have risen significantly upon entering the general circulation due to its relatively short half life (112).

One cannot discuss the role of butyrate *in vivo* without discussing dietary fiber. There is consistent epidemiological and experimental evidence supporting the protective effect of dietary fiber against risk of colorectal cancers. Using rodents as the experimental model, many studies on the effect of dietary fibers on carcinogen-induced colon cancer have been published. Wheat bran is the most widely studied fiber source. It appears that in the majority of the experiments in which wheat bran was fed to the rodents there was evidence of decreased tumor development (113-115). Epidemiological and animal studies suggest that poorly fermented fibers (e.g. wheat bran) are protective against colon cancer, whereas the readily fermented fibers (e.g. oat bran) are not protective and may possibly be promotive of tumor development (4-7). The differences observed in the colon cancer incidence with ingestion of different dietary fibers are due, at least in part, to the fact that dietary fibers are not a single entity. They are a complex group of substances with distinct physicochemical properties, and thus, exert different effects within the colon.

It is evident in the majority of rodent studies using carcinogens that the distal colon is the primary site of tumor occurrence (23). Typically, the distal colon has the lowest concentration of SCFA, including butyrate, compare with the cecum and proximal colon that are the primary sites of fiber fermentation. Thus, if butyrate is protective, it seems reasonable to suggest that diets generating the greatest concentrations of butyrate in the distal colon would be more desirable. However, diets containing oat bran produced larger concentrations of SCFA, including butyrate, in both proximal and distal colon, but had a significantly higher incidence of colon tumors than their wheat bran counterparts in a chemically induced rat

tumor model (23) The data suggests that distal colonic butyrate concentrations alone are not protective of colonic tumor development. Wheat bran is a complex fiber that contains many morphologic components, such as, aleurone and the pericarp seed-coat, and a number of other substances, such as phytates and phytosterols, that may have antineoplastic properties of their own (116,117).

From our studies, it is evident that butyrate demonstrates antiproliferative effect on *APC* defective colon adenocarcinoma cell lines *in vitro*, but it does not show any change in the tumor incidence *in vivo* using an experimental animal model with an *Apc* gene defect. Recently, it has been reported that the lipid fraction, not the phytate, or fiber fractions of wheat bran exerts the strong inhibitory effect against both colon (45%-65% inhibition) and mammary carcinogenesis (35% inhibition) and that these effects were related to reductions in the expression and activities of cyclooxygenase-2 (COX-2) and inducible nitric oxide synthase (iNOS) (118). Our results and the results of others indicate that butyrate may not have the same effect *in vivo* as observed *in vitro*. Our study further supports the hypothesis that butyrate is an important modifier or regulator of tumor cell growth *in vitro*, but its role *in vivo* has yet to be clearly established. The differences observed *in vivo* and *in vitro* may be a result of culture condition or due to differential sensitivity of normal and tumor cells to butyrate. It is possible that normal and tumor cells respond differently to butyrate. It is assumed that the shift from aerobic to anaerobic metabolism which is a characteristic of carcinoma of colon, may lead to increased intracellular levels of butyrate and causes growth inhibition. Recently, it has been demonstrated by Williams et al. that adjacent non-neoplastic

cells can have dramatic influences on tumor growth *in vivo* that cannot be duplicated *in vitro* (119). There are other lines of evidence suggesting that the growth inhibitory or growth stimulatory effect of butyrate may depend on the availability of other energy sources (120). The preferred energy source for the colonic epithelium *in vivo* is butyrate, whereas most cell cultures are grown in tissue culture medium containing high levels of glucose for their energy (120). It has been found that butyrate shows growth stimulatory effect in glucose-depleted culture conditions at relatively low concentrations (0.5mM to 2mM), whereas in the presence of glucose these same concentrations of butyrate induced apoptosis. These observations may, in part, provide an explanation for the apparent contradictory effects of butyrate on Proliferation reported *in vivo* and *in vitro*.

PART VI

CONCLUSION

The overall objective of this thesis is to determine if butyrate has anti-tumor properties using *in vitro* and *in vivo* intestinal tumor models with defects in the *APC* gene. To evaluate the efficacy of butyrate *in vitro*, human colonic adenocarcinoma cell lines that possessed mutations in the *APC* gene were used in the experiments. *Apc^{Mm/+}* mice model was used to investigate the anticarcinogenic properties of tributyrin on tumor incidence *in vivo*. Over the last several years *Apc^{Mm/+}* mice has been used to evaluate the nutrition intervention on intestinal tumorigenesis because of its germline mutation in the murine *Apc* gene (27). The *Apc^{Mm/+}* mice is highly susceptible to tumor formation and are genotypically and phenotypically similar to human FAP (26).

It has been demonstrated from the *in vitro* studies that butyrate has antiproliferative effect on colonic tumor cell lines. Butyrate inhibited growth of SW480, SW620 and DLD-1 cell lines in a dose-dependent manner with marked reductions of 50-96% at doses between 2.5mM to 10mM, similar to the intracolonic concentrations found *in vivo*. However, the dietary supplementation of 3% (w/w) TB fed to *Apc^{Mm/+}* mice for 7 weeks failed to demonstrate any clinical efficacy of butyrate on the tumor incidence in the colon or small intestines. It is evident from the report that butyrate may not have the same effect *in vivo* as observed *in vitro*. The data further supports the hypothesis that butyrate is an important modifier of tumor cell growth *in vitro*, but its role as an anti-tumor metabolite *in vivo* needs to be further investigated.

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APPENDIX I

Significance level of different concentration* of NaB on SW480 cell growth

Cell Line	Conc. Of NaB (mM)	df	F	P-value
SW480 Day 1	2.5	1,6	10.02	0.019
	5	1,6	6.36	0.045
	10	1,6	6.41	0.044
SW480 ³ Day 2	0.125	1,6	9.27	0.022
	1	1,6	7.643	0.032
	2.5	1,6	92.04	7.33E-05
	5	1,6	74.787	0.001
	10	1,6	30.07	0.001
SW480 Day 4	0.5	1,6	9.73	0.02
	1	1,6	36.58	0.009
	2.5	1,6	100.52	5.70672E-05
	5	1,6	153.67	1.68179E-05
	10	1,6	173.05	1.19082E-05

Critical value of F at df (1,6) and $p < 0.05 = 5.99$

* Only statistically significant values are shown

APPENDIX II

Significance level of different concentration* of NaB on SW620 cell growth

Cell Line	Conc. Of NaB (mM)	df	F	P-value
Sw620 Day 1	0.5	1,6	33.39	0.001
	2.5	1,6	6.28	0.0046
	5	1,6	23.05	0.002
	10	1,6	26.93	0.002
SW620 Day 2	0.5	1,6	41.35	0.0006
	1	1,6	6.45	0.0439
	2.5	1,6	87.41	8.4871E-05
	5	1,6	332	1.75976E-06
	10	1,6	332	1.75976E-06
SW620 Day 4	0.125	1,6	8.35	0.027
	0.250	1,6	11.45	0.014
	0.5	1,6	17.37	0.005
	1	1,6	21.90	0.003
	2.5	1,6	34.25	0.001
	5	1,6	40.81	0.0069
	10	1,6	47.34	0.0004

Critical value of F at df (1,6) and $p < 0.05 = 5.99$

* Only statistically significant values are shown

APPENDIX III

Significance level of different concentration* of NaB on DLD1 cell growth

Cell Line	Conc. Of NaB (NaB)	df	F	P-value
DLD-1 Day 1	5	1,6	94 47	6 81E-05
DLD-1 Day-2	0 125	1,6	8 89	00 024
DLD-1 Day-4	2 5	1,6	17 54	0 005
	5	1,6	27 81	0 001
	10	1,6	58 61	0 002

Critical value of F at df (1,6) and $p < 0.05 = 5.99$

* Only statistically significant values are shown

VITA

Sonia Shahnaz was born in Chittagong City, Bangladesh on October 11, 1968. She is the eldest daughter of Mr. Ataur Rahman and Mrs. Taslima Rahman. She grew up in the city of Dhaka, where she attended her school and college. She received her Secondary School Certificate (S.S.C) in 1984 and her Higher Secondary School Certificate (H.S.C) in 1986 from Dhaka Board of Education and attended Dhaka University afterwards where she received the Degree of Bachelor of Science in Biochemistry in 1993 and her Master's degree (also in Biochemistry) in 1994.

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She is a member of Bangladesh Biochemical Association. When attending in Dhaka University she was engaged in different cultural activities and held several offices. She is an amateur artist and musician.