MECHANISMS OF APOPTOSIS OF COLORECTAL CANCER CELLS BY BUTYRATE AND OTHER PREVENTIVE AGENTS

A thesis submitted to the University of Adelaide as the requirement for the degree of Doctor of Philosophy

by

Fugui Chai, M. Med.

Department of Medicine, The University of Adelaide,
The Queen Elizabeth Hospital

August 2001

TABLE OF CONTENTS

LIST OF MINOR CORRECTIONS IS FOUND ON THE LAST TWO PAGES

ABSTRACT

Dietary and chemopreventive strategies are important to reduce the frequency of colorectal cancer. The mechanism of the prevention remains to be elucidated. The main aims of this thesis were: 1) to study the mechanism by which butyrate (the main fermentation product of dietary fibre), induces apoptosis of colorectal cancer (CRC) cells, and 2) to determine the effect of butyrate in combination with NSAIDs (chemopreventive agents) and other agents on apoptosis of CRC cells. For these studies, LIM1215 cells and, in some experiments, SW480 cells were used.

DEVD-caspase activation was involved in the apoptosis of CRC cells by various dietary and chemopreventive agents. The DEVD-caspase activity was generally in close agreement with DNA fragmentation, morphological changes and flow cytometric detection, all markers of apoptosis. However, inhibition of DEVD-caspase activity in the cells by caspase inhibitors only partially suppressed DNA fragmentation and morphological changes of apoptosis. This was found not only for butyrate, but also for the zinc chelator N,N,N',N'-tetrakis (2-pyridylmethyl)-ethylenediamine (TPEN), sulindac sulphide and sulindac sulfone in LIM 1215 cells.

The effects of butyrate on growth arrest and apoptosis paralleled in time the shift in the phosphorylation state of the cell-cycle regulatory retinoblastoma protein (pRb) protein. They were also accompanied by cleavage of $p21^{Waf1/Cip1}$ (p21), a potential caspase-3 target during apoptosis. The cleavage product of p21, a 15KDa polypeptide (p15) first appeared immediately after onset of DEVD-caspase activity in butyrate-treated

LIM1215 cells and increased thereafter. A search of the primary sequence of human p21 revealed a likely caspase cleavage motif DHVD at a position in the protein that would roughly correspond to the p15 fragment. Following this observation, a number of papers appeared in the literature also showing that p21 is cleaved by caspase-3.

Overexpression of p21 or p15 in LIM1215 or SW480 cells by transient transfection had no effects on caspase activation or apoptosis. However, this system had its limitations, since overexpression of p21 and p15 appeared to growth arrest the cells, making interpretation of the results difficult.

CRC cells could be rendered butyrate-resistant by three cycles of butyrate treatment. The resistance to butyrate had the following main characteristics: 1) cells were also resistant to a second, structurally-unrelated inhibitor of histone deacetylase trichostatin A; 2) the expression of p21, was very high in the resistant cells; 3) p15 was formed when sensitive cells were treated with butyrate but not when resistant cells were treated with butyrate; and 4) butyrate-resistant cells remained sensitive to caspase-activation by staurosporine, suggesting that butyrate-resistance occurs upstream of activation of caspase-3.

It is likely that butyrate is inducing some anti-apoptotic factor(s). This could be a factor like p21, Cox or Lox which have their own distinct functions but also exert an anti-apoptotic effect. There may be multiple factors involved in the resistance. However, both Cox inhibitor sulindac sulfide and Lox inhibitor nordihydroguaiaretic acid (NDGA) did not overcome the resistance to butyrate. The cross-resistance to sulindac compounds may have therapeutic implications.

Both sulindac sulfide (which inhibits Cox) and sulindac sulfone (which does not inhibit Cox) induced apoptosis of CRC cells in concentration- and time-dependent ways, suggesting sulindac-induced apoptosis is not dependent on inhibition of Cox. Further supporting this hypothesis, the interaction between sulindac sulfide and arachidonic acid on activation of caspase-3 was synergistic rather than additive and SW480 cells lacking Cox-2 still underwent apoptosis.

Butyrate was found to act synergistically with sulindac sulfide, sulindac sulfone, NDGA and arachidonic acid. This may be important in chemoprevention of colorectal cancer where a combination of sulindac and high dietary fibre may yield better effects than either agent alone.

Experiments to investigate the involvement of zinc in the apoptosis of LIM1215 cells led to the following major conclusions: 1) apoptosis occurs in zinc-depleted cells and is accompanied by activation of caspase-3 and caspase-6; 2) zinc depletion enhances butyrate-induced apoptosis while zinc supplementation suppresses it; and 3) activation of caspase-3 by zinc depletion is rapidly followed by loss of p21, first to a 15KDa fragment which is then further degraded.

Another dietary factor phytic acid killed CRC cells in vitro by caspase-3-dependent apoptosis. Although phytic acid is a zinc chelator in vivo, apoptosis was not mediated by depletion of intracellular zinc. A combination of butyrate and phytic acid resulted in additive effects on induction of DEVD-caspase activity. A model describing these interactions is shown in Fig 10.1.

In conclusion, the experiments described in this thesis have provided new data on the caspase-3 activation and apoptosis in butyrate-induced apoptosis of CRC cells, and the

interaction of these processes with a number of cellular factors and pharmacological agents.

DECLARATION

This work contains no material which has been accepted for the award of any other degree or diploma in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text.

I give consent to this copy of my thesis, when deposited in the University Library, being available for loan and photocopying.

August 2001

ACKNOWLEDGEMENTS

I would like to thank the University of Adelaide for offering me an International Postgraduate Research Scholarship (IPRS) and the University of Adelaide Scholarship (UAS), which made it possible for me to complete my Ph D program.

I wish to thank everyone who helped me in my PhD candidature. Firstly, I am sincerely grateful to my two principal supervisors, Dr Peter D Zalewski and Professor Graeme P Young for their patient guidance and encouragement during this project and for many interesting discussions and for their support in preparation of this thesis. I would also like to express my appreciation to Professor Young, for his recommendation in my application for scholarships and financial support. I would like to thank my third supervisor, Dr Andreas Evdokiou, for his assistance with transfection experiments. All of their supervision and encouragement were crucial for completion of this thesis.

I wish to thank Dr Alexander Dobrovic, the postgraduate co-ordinator and Professor Richard Ruffin, the head of Department of Medicine, The Queen Elizabeth Hospital, for their support during my research.

I would also thank Drs Andrew Zannettino and Liza Raggatt, for their help in FACS analysis, and the following people for their support and advice through the years: Sarah Appleton, Joanne Carter, Lien Ho, Austin Milton, Ermioni Mourtzios and Ai Truong-Tran.

I am indebted to Professor Shirong Li, the head of Department of Gastrenterology, Beijing Millitary General Hospital for giving me the opportunity to come to Australia to undertake this PhD program.

Finally, I would like to thank my parents and my daughter for their continued support and encouragement.

PUBLICATIONS

1. **Chai F.**, Evdokiou A., Young G.P., Zalewski P.D. Relationship of p21Waf1/Cip1 and pRb to butyrate-induced apoptosis of LIM 1215 colorectal cancer cells. *Journal of Gastroentorology and Hepatology* 1998 (Suppl): A 190 (Abstract)

2. **Chai F.**, Truong-Tran A.Q., Ho L.H., Zalewski P.D. Regulation of caspase activation and apoptosis by cellullar zinc deprivation: a review. *Immunology and Cell Biology* 1999; 77, 272-278

3. Young GP, **Chai F.** and Zalewski P.D. Polysaccride fermentation, butyrate and apoptosis in the colonic epithelium. *Asia Pacific J Clin Nutr* 1999; 8(Suppl):27S-31

4. **Chai F.**, Evdokiou A., Young G.P., Zalewski P.D. Involvement of $p21^{Waf1/Cip1}$ and its cleavage by DEVD-caspase in apoptosis of colorectal cancer cells by the colonic fermentation product butyrate. *Carcinogenesis* 2000; 21:7-14

5. Young G.P., **Chai F.**, Zalewski P.D. *Primary prevention of colorectal cancer*. In: Li SR, ed. Colorectal Cancer--Early Diagnosis, Therapy and Prevention. Beijing: Science Publishing House, 2000: 365-380.

6. Truong-Tran AQ, Ho LH, **Chai F**, Zalewski P.D. Cellular zinc fluxes and the regulation of Apoptosis/Gene-directed cell death. *J Nutr* 2000; 130(5 Suppl): 1459S-66S.

7. **Chai F.**, Truong-Tran A.Q., Evdokiou A., Young G.P., Zalewski P.D. Intracellular zinc depletion induces caspase activation and $p21^{Waf1/Cip1}$ cleavage in human epithelial cell lines. *J Infect Disease* 2000; 182 (S1): S85-S92.

8. **Chai F.**, Young G.P., Zalewski P.D. Butyrate-resistant colorectal cancer cells are also resistant to sulindac sulfide and sulfone. *Gut* 2000; 47 (suppl III): A42 (Abstract).

9. **Chai F.**, Evdokiou A., Young G.P., Zalewski P.D. Effect of inhibitors of cyclooxygenease-2 and lipoxygenase on butyrate-induced apoptosis of colorectal cancer cell lines. (in preparation)

10. Betts W.H., Zhang C.L., **Chai F.**, Zalewski P.D. Lyprinol-induced apoptosis in cancer cells. (submitted to Carcinogenesis)

CONFERENCE PRESENTATIONS

Chai F., Evdokiou A., Young G.P., Zalewski P.D. Relationship of p21Waf1/Cip1 and pRb to butyrate-induced apoptosis of LIM 1215 colorectal cancer cells. Australian Gastroenterology Week 1998. Plenary oral presentation, young investigator awards candidate.

Chai F., Evdokiou A., Young G.P., Zalewski P.D. Involvement of p21Waf1 and its cleavage by caspase-3 in apoptosis of colorectal cancer cells by the colonic fermentation product butyrate. American Digestive Disease Week 1999. Poster presentation.

Chai F., Evdokiou A., Young G.P., Zalewski P.D. The effect of zinc depletion on apoptosis in colorectal cancer cells. American Digestive Disease Week 2000. Poster presentation.

Chai F., Young G.P., Zalewski P.D. Butyrate-resistant colorectal cells are also resistant to sulindac sulfide and sulfone. "8th United European Gastroenterology Week, 2000". Oral presentation.

LIST OF ABBREVIATIONS

AA	arachidonic acid
AIF	apoptosis-inducing factor
AOM	azoxymethane
APC	adenomatous polyposis coli tumor-suppressor gene
CAD	caspase activated DNase
CDK	cyclin dependent kinase
CEA	carcinoembryonic antigen
CRC	colorectal cancer
Cox	cyclooxygenase
zDEVD-AFC	z-asp-glu-val-asp-7-amino-4-trifluoro-methyl-coumarin
zDEVD-fmk	z-asp-glu-val-asp-fluoro-methyl-ketone
DFF	DNA fragmentation factor
DMAB	3,2'-dimethyl-4-aminobiphenyl
DMSO	Dimethyl sulfoxide
EGFP	enhanced green fluorescent protein
FADD	Fas-associated death domain
FAP	familial adenomatous polyposis
FBS	foetal bovine serum
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
GSK-3	glycogen synthase kinase –3

HETEs	hydroxyeicosatetraenoic acids
HNPCC	hereditary nonpolyposis colorectal cancer
ICAD	inhibitor of CAD
IP_6	inositol hexaphosphate,
Lox	lipoxygenase
LTA4	leukotriene A4
LTB4	leukotriene B4
MMR	DNA mismatch repair
MSI	microsatellite instability
MCS	multiple cloning site
MPT	mitochondrial permeability transition
NDGA	nordihydroguaiaretic acid
NSAIDs	non-steroidal anti-inflammatory drugs
PCR	Polymerase chain reaction
p21	$p21^{Waf1/Cip1}$
PPAR	peroxisome proliferator-activated receptor
PBS	phosphate buffered saline
Rb	retinoblastoma gene product
RIE	Rat intestinal epithelial
STS	staurosporine
TCF-4	transcription factor T cell factor 4
TPA	12-O-tetradecanoylphorbol-13-acetate
TBS	tris-buffered saline

TNF	tumor necrosis factor
TSA	trichostatin A
TUNEL	terminal transferase mediated DNA nick end labelling
zVAD-fmk	z-val-ala-asp-fluoro-methyl-ketone
VEID-AFC	val-glu-ile-asp-7-amino-4-trifluoro-methyl-coumarin

CHAPTER 1

LITERATURE REVIEW

1.1 GENERAL INTRODUCTION

This chapter begins by briefly reviewing colorectal cancer and its prevention. It then goes on to an overview of apoptosis, including some of the regulatory molecules, and in particular $p21^{Waf1/Cip1}$ (p21). The review then focuses on the mechanisms of action of butyrate and non-steroidal anti-inflammatory drugs (NSAIDs) on apoptosis of colorectal cancer cells, as well as the modulatory effects of zinc and phytic acid.

1.2 COLORECTAL CANCER AND PREVENTION

1.2.1 Epidemiology of colorectal cancer

Cancer of the colon and rectum (colorectal cancer) is a major cause of cancer-associated morbidity and mortality in North America, Europe, and other regions with similar life-style and dietary habits. Globally, it is the third most common cancer (Silverberg & Lubera, 1989). The incidence of colorectal cancer is remarkably different between different populations. Incidence rates are highest in the Westernised countries of North America, Australia, and New Zealand, intermediate in areas of Europe, and low in regions of Asia, South America, and especially sub-Saharan Africa (Parkin, et al., 1985; Parkin, et al., 1992). There were an estimated 138,200 cases of colorectal cancer in the USA in 1995 with 55,300 deaths (Wingo et al, 1995). In the United Kingdom, 19,000 deaths are reported annually. In Australia (New South Wales), the incidence rate in males is 23/100,000 and in females is 20.1/100,000. In China (Shanghai), the incidence rate in males is 8.5/100,000, and in females is 7.6/100,000.

1.2.2 The genetics of colorectal cancer

Recently, developments in genetics, molecular biology, and epidemiology have increased our understanding of the role of genes in the aetiology of colorectal cancer. The development of colorectal cancer is a multistep process involving the accumulation of genetic changes. Mutations in at least four or five genes are required for the formation of a malignant tumour. The major genetic events involved in colorectal tumorigenesis, according to a model proposed by Fearon and Vogelstein (1990), are the mutations of adenomatous polyposis coli tumor-suppressor gene (APC) in early stage of development, oncogenic k-Ras mutations in adenomatous stage, and p53 mutations and deletions of chromosome 18q (DCC) in the transition to malignancy (Fearon and Vogelstein, 1990). Subsequent studies have identified additional genetic events as well as the specific molecular pathways perturbed by each of these mutations. These pathways regulate important cellular processes in neoplastic cells including proliferation and apoptosis. It is known there are at least two completely different pathogenetic pathways for colorectal cancer.

The first is the APC/β–catenin pathway. The APC gene product is able not only to bind to itself forming oligomers but is also able to bind to many intracellular proteins including β-catenin, γ-catenin, glycogen synthase kinase (GSK)-3, axin, tubulin, EB1, and hDLG (Kinzler and Vogelstein, 1996). Together with GSK-3, APC binds and targets cytoplasmic β-catenin for degradation via a ubiquitin-mediated proteasomal pathway (Rubinfeld *et al.*, 1996). Truncation of APC results in the disruption of complex formation and ultimately increased cytoplasmic levels of free β-catenin. Free β-catenin is translocated to the nucleus, where it initiates transcription of genes after interacting with transcription

factor T cell factor 4 (TCF-4). It is known that TCF-4 up-regulates the oncogenes c-Myc and cyclin D1, as well as matrilysin, c-jun, fra-1, and urokinase-type plasminogen activator receptor (Crawford *et al.*, 1999; He *et al.*, 1999; Mann *et al.*, 1999; Tetsu and McCormick, 1999). Another recently identified target is the peroxisome proliferator-activated receptor δ (PPARδ) gene, which is also regulated by NSAIDs (He *et al.*, 1999). Germ line mutations in APC result in either familial adenomatous polyposis or Gardner's syndrome. Subsequent studies have suggested that the APC tumor-suppressor gene also plays a critical early role in sporadic colon cancer, and APC is mutated in more than 70% of all colorectal cancers. This high mutational rate is unique to colorectal cancer.

The second pathogenic pathway for colorectal cancer involves inactivation of the DNA mismatch repair (MMR) system, a complex enzymatic proof-reading system that corrects base pair mismatches that arise during DNA replication. DNA mismatch repair gene defects are presumed to lead to accumulation of widespread mutations within short repetitive sequences. Because most stretches of microsatellite DNA lie in noncoding sequences, many such alterations are unlikely to be functionally significant. However, mutations also occur in coding exons of genes with repetitive sequences involved in the regulation of cell growth, such as those encoding the type II TGF- receptor (Markowitz *et al.*, 1995), cell cycle regulated transcription factor E2F-4 (Yoshitaka *et al.*, 1996), T-cell factor 4 (Duval *et al.*, 1999), β–catenin (Salahshor *et al.*, 1999), and Bax (involved in apoptosis) (Duval *et al.*, 1999); even some of the DNA MMR genes (hMSH6 and hMSH3) are affected (Malkhosyan *et al.*, 1996). Some of these mutations lead to loss of function in tumour suppressor genes (i.e. the type II TGF- receptor gene, Bax, and the MMR genes), and other mutations cause gain of function in an oncogene (i.e., β–catenin). Therefore, loss

of DNA MMR activity results in the accumulation of a very large number of mutations, which eventually disturbs cell growth (Boland *et al.*, 1998).

Germline mutations in DNA MMR genes cause hereditary nonpolyposis colorectal cancer (HNPCC), making up about 5-10% of all colorectal cancers (Thorson *et al.*, 1999). Mutations in five mismatch repair genes (hMSH2, hMSH6, hMLH1, hPMS1, and hPMS2) have been described to date, which have now been thought to be associated with the HNPCC (Chung and Rustgi, 1995). Each of the five genes encodes a protein involved in DNA mismatch repair. As much as 45%-70% of HNPCC families have mutations in one of these genes, most frequently either hMSH2 or hMLH1 (Liu *et al.*, 1996; Wijnen *et al.*, 1998).

One of the hallmarks of defective DNA MMR system is microsatellite instability (MSI). All HNPCC tumours display MSI and about 15% of all colorectal colorectal cancers have MSI. More than 80% of sporadic colorectal tumours with MSI, show hypermethylation of CpG sites in the promoter for the hMLH1 gene, suggesting that this event may be a primary mechanism underlying the MSI phenotype in sporadic colorectal tumours (Boland *et al.*, 2000). In both the hereditary and nonhereditary settings, the same target genes are inactivated, usually at the repetitive sequences mentioned previously.

Since the development of colorectal cancer is a multistep process, which is prolonged in time (approximately 5-10 years on average in humans), it is clearly possible to block or reverse the pathological events at different steps of carcinogenesis. It is by altering the precancer biology that dietary factors and chemopreventive agents exert their protective effects in the prevention of colorectal cancer.

1.2.3 Prevention of colorectal cancer

1.2.3.1 Dietary prevention of colorectal cancer

Environmental mutagenic factors may determine which susceptible individuals develop carcinomas. Environmental risk factors for colorectal cancer are found in a Western diet, rich in fat, meat, and animal protein and low in fibre, fruit, and vegetables. There is a strong relationship between per capita intake of fat whether it is measured as total fat, cholesterol, or saturated fatty acid, and the incidence of colorectal cancer. This finding has been seen in most case-control studies (Haenszel *et al.*, 1973; La Vecchia *et al.*, 1988; Lee *et al.*, 1989; Manousos *et al.*, 1983; Young and Wolf, 1988) and several prospective studies (Giovannucci *et al.*, 1994; Willett *et al.*, 1990).

Fatty acids particularly in the ionized form have been shown to result in increased mucosal proliferation. Another mechanism by which fat enhances tumorigenesis is by stimulating bile acid flow and increasing fecal bile and neutral steroid concentrations (Hill, 1977; Reddy, 1981). Fat stimulates the release of secondary bile acids (deoxycholic acid). The secondary bile acids increase cellular proliferation by causing nonspecific mucosal damage as well as producing oxygen radicals which in turn stimulate mucosal proliferation.

Meat, especially red meat, is believed to be associated with increased risk. The American Institute for Cancer Research and the World Cancer Research Fund (World-Cancer-Research-Fund, 1997) suggested that red meat was associated with higher risk than fat. Another study in the literature also concluded that diets with less red meat are associated with reduced risk of colorectal cancer (Committee-on-Medical-Aspects-of-Food, 1998). Nevertheless, a recent meta analysis of five cohorts showed that meat eaters were not at greater risk than non-meat eaters (Key *et al.*, 1998). One explanation for the hypothesis

that red meat is a risk factor is that mutagenic heterocyclic amines are produced during cooking of meat at high temperature (Gerhardsson de Verdier *et al.*, 1991). Studies in rats and mice have shown that heterocyclic amines induce tumours particularly in the colon and mammary gland, in a dose-dependent way (Bogen, 1994; Ito *et al.*, 1991; Nagao *et al.*, 1994). The metabolic activation of food-borne heterocyclic amines to colon carcinogens in humans is hypothesised to occur via N-oxidation, followed by O-acetylation to form the N-acetoxy arylamine. N-acetoxy arylamine can bind to DNA to give carcinogen-DNA adducts. These steps are catalysed by hepatic cytochrome P4501A2 (CYP1A2) and acetyltransferase-2 (NAT-2), respectively, which are known to be polymorphic in humans. On the basis of this proposed metabolic activation pathway, patients at greatest risk to develop colorectal cancer or nonfamilial polyps should be those who possess both the rapid NAT-2 and rapid CYP1A2 phenotypes and are exposed to high dietary levels of carcinogenic heterocyclic amines. In support of this hypothesis, the risk of adenoma or cancer increased with more intake of meat in fast but not in slow acetylators (Roberts Thomson *et al.*, 1996). Thus, prolonged overcooking and charring of the surface of the meat should be avoided.

Therefore, the most consistent protective benefit is by reducing the dietary intake of fat and red meat and increasing fibre. Prevention of colorectal cancer by fibre is probably the best known.

The fibre hypothesis was proposed by Burkitt, who observed that African natives had low colon cancer rates and high fecal bulk (Burkitt, et al., 1971). He presumed that the low colon cancer rates and high fecal bulk were due to a high fibre diet. Howe et al (Howe *et al.*, 1992) analysed 13 case-control studies. 12 of them supported the hypothesis that

dietary intake of fibre decreased risk of cancers of the colon and rectum; this inverse association with fibre is similar in magnitude for left- and right-sided colon and rectal cancers, for men and for women, and for different age groups. The authors concluded that colon cancer in the United States could decrease by 31% by an average increase in fibre intake from food sources of about 13 g/day. A meta-analysis of 60 studies supported the hypothesis that fibre protects against the colon cancer (Trock *et al.*, 1990). Evidence from another analysis has also supported this protective action of dietary fibre (Potter, 1996). However, not all studies supported this fibre hypothesis. Two large cohort studies in the United States failed to find a protective effect of fibre (Giovannucci *et al.*, 1994) (Willett *et al.*, 1990).

Numerous mechanisms by which fibre could inhibit the development of colorectal cancer have been proposed. Firstly, fibre can increase fecal weight, shorten transit time (and therefore less contact time between tumor promoters in the lumen and the colonic mucosa). It can also dilute colonic contents, including, presumably, carcinogenic agents (Burkitt, et al., 1971). Secondly, fibre can not only inhibit hydroxylation of bile acids and regulate energy intake (Jacobs, 1988) but also absorb bile salts, which are known promotional agents for colorectal cancer (Kamano *et al.*, 1999; Nagengast *et al.*, 1995). Finally, fermentation of fibre by bacteria can also produce short chain fatty acids, lowering the pH and creating an environment which is less conducive to cancer development (Cummings, 1983; Nyman & Asp, 1982).

Many animal studies have been designed to test the fibre hypothesis. Dietary wheat bran at levels of 15% plus 5% dietary fat decreased the incidence (number of animals with tumour) and multiplicity (number of tumours/tumour-bearing rats) of small intestine and

colon tumours induced by azoxymethane (AOM) (Reddy *et al.*, 1981) or 3,2' –dimethyl-4-aminobiphenyl (DMAB) (Reddy and Mori, 1981) in male F344 rats, while dietary corn bran increased the incidence and multiplicity of colon tumours induced by DMAB (Reddy *et al.*, 1983). Similar protective effects by wheat bran (Barbolt and Abraham, 1978), but not pectin (Freeman *et al.*, 1980), were observed in rats which had chemically induced colorectal cancer. Thus not only the amount but also type of fibre play an important role in increasing or decreasing colon carcinogenesis in animal models.

Dietary fibre is heterogeneous but is largely comprised of non-starch polysaccharides and non-carbohydrates. Animal studies showed that not all forms of fibre could give the same protection. Certain fibre polymers may be beneficial, whereas others may increase susceptibility to chemically-induced colon cancer. In general, fibres that are more insoluble and less fermentable such as lignin and cellulose tend to protect against tumor development, whereas more soluble and fermentable fibres such as pectins and gum increase tumor production in animal carcinogenesis experiments (Jacobs, 1986). Wheat bran has inhibited cancer development in most studies, but not bran from corn, rice, oats, and soybean. Probably fibre from wheat may be preferred to that from other grains because it is fermented to short chain fatty acid butyrate in large amounts (McIntyre, 1993). The protection of butyrate again colorectal cancer will be reviewed in 1.5.

Recently, there were three randomised clinical trials reported to test the effects of fibre supplement on prevention of colorectal adenoma recurrence (Alberts *et al.*, 2000; Schatzkin *et al.*, 2000). Data from these studies did not support the hypothesis that a dietary supplement of wheat-bran fibre protected against recurrent colorectal adenomas. In Schatzkin's study (Schatzkin *et al.*, 2000), 2079 men and women (≥35 years) were

randomly assigned to two groups, who had had one or more histologically confirmed colorectal adenomas removed within six months. A total of 1905 subjects (91.6%) completed the study. 958 subjects in the intervention group were given a diet low in fat (20 percent of total calories) and high in fibre (18 g of dietary fibre per 1000 kcal), fruits and vegetables (3.5 servings per 1000 kcal); the 947 in the control group were given a standard brochure on healthy eating and assigned to follow their usual diet. After one and four years follow-up with colonoscopy, findings were that 39.7% in the intervention group and 39.5% in the control group had at least one recurrent adenoma. Among subjects with recurrent adenomas, the mean (±SE) number of such lesions was 1.85±0.08 in the intervention group and 1.84±0.07 in the control group. The rate of recurrence of large adenomas (with a maximal diameter of at least 1 cm) and advanced adenomas (defined as lesions that had a maximal diameter of at least 1 cm or at least 25% villous elements or evidence of high-grade dysplasia, including carcinoma) did not differ significantly between the two groups. All these findings shows that a diet low in fat and high in fibre, fruits, and vegetables does not influence the risk of recurrence of colorectal adenomas. Similar results were found in Alberts' trial showing that a dietary supplement of wheat-bran fibre does not protect against recurrent colorectal adenomas.

Besides fibre, resistant starch escaping digestion in small intestine and entering the large bowel can also be anaerobically fermented into short chain fatty acids: acetate, propionate and butyrate (Phillips *et al.*, 1995). Both increase of butyrate and decrease of pH due to the fermentation in colon are likely to be protective against colorectal cancer (Muir *et al.*, 1998). Data from epidemiological studies supported the hypothesis that resistant starch protected against colorectal cancer (Cassidy *et al.*, 1994), while rat studies failed to

be supportive (Young *et al.*, 1996). The high starch intake in China is associated with a low colorectal cancer (Cassidy *et al.*, 1994). It has been suggested that this might be due to resistant starch but a recent study has not shown the Chinese diet to contain significant amounts of resistant starch (Muir *et al.*, 1998).

Several micronutrients have been investigated in relation to risk of colorectal cancer. They are dietary calcium and vitamin D, folate and some antioxidants including β–carotene, vitamin E and C and selenium (Shike *et al.*, 1990). Early studies suggested that a high calcium diet provided modest protection against colorectal cancer possibly by inhibiting proliferation of colonic epithelium (Hyman *et al.*, 1998; Sitrin *et al.*, 1991; Wargovich *et al.*, 1983), however, epidemiological analyses are inconsistent (Bergsma Kadijk *et al.*, 1996; Martinez *et al.*, 1996). In a clinical trial, neither β–carotene nor the combination of vitamin E and C reduced the incidence of colon polyps (Greenberg *et al.*, 1994). Populations deficient in selenium have an increased incidence and mortality from colorectal cancer (Nelson *et al.*, 1996). A double blind, randomised, placebo-controlled cancer prevention trial was designed to test if selenium supplementation decreased the incidence of carcinoma of the skin. Incidentally, it was found that the group receiving selenium supplementation had a 40% decrease in colorectal cancer (8 colorectal cancers in the selenium group versus 19 in the placebo group ($p=0.03$) over 8271 person-years) (Clark *et al.*, 1996). The dose of 200 μg per day was estimated to be approximately twice the projected typical dietary intake of these patients and was three to four times the recommended daily allowance. This effect requires confirmation in an independent trial of appropriate design before public health recommendations regarding selenium supplementation should be made.

Folate is rich in wheat bran, baker's yeast, cruciferous vegetables and spinach (Briggs and Wahlqvist, 1998). Low folate diets are a risk for colorectal cancer especially in habitual alcohol consumers but there is no evidence that excessive folate consumption is protective (Kune, 1996).

1.2.3.2. Chemoprevention of colorectal cancer

Chemoprevention refers to the use of natural or synthetic agents to reverse, suppress, or prevent progression or recurrence of cancer (Bresalier, 2000). Because there are at least two separate and independent pathways to carcinogenesis in the colon, one must consider that an effective preventive strategy for one pathway may not work for another (Boland *et al.*, 2000). The forms of genomic instability involved in these two pathways are completely distinct from one another. The common pathway observed in about 85% of colorectal neoplasms (Fig 1.1A) requires a process in which chromosomal instability results in losses of tumor-suppressor genes. The mechanism responsible for this type of genomic instability is unknown, which limits our ability to develop preventive strategies for these neoplasms. The second pathway, MSI is found in the remaining 15% of colorectal neoplasms (Fig 1.1B), and is better understood. So far, NSAIDs have been the most widely studied and confirmed effective chemopreventive agents in colorectal cancer, particularly in familial adenomatous polyposis (FAP).

Waddell (Waddell and Loughry, 1983) reported the first human studies showing that an NSAID (sulindac) caused regression of, and prevented recurrence of, adenomatous colorectal polyps in Gardner's syndrome. Since then, there have been several controlled clinical trials confirming Waddell's observations and demonstrating the therapeutic effect of sulindac on precancerous lesions in FAP patients (Waddell *et al.*, 1989; Giardiello *et al.*,

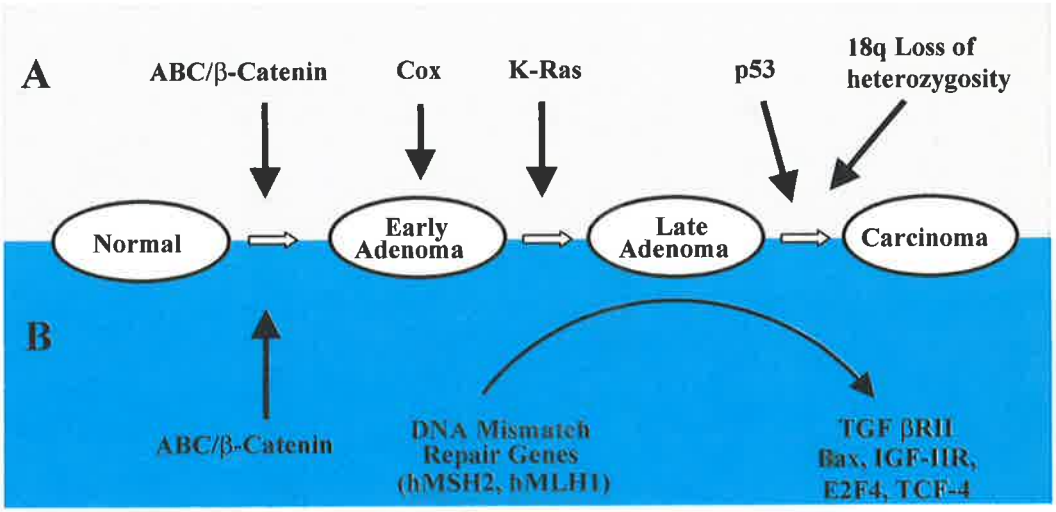


Figure 1.1 Overall scheme of key genetic events in colorectal tumorigenesis. The critical genes involved in the development of colorectal polyps and cancer are illustrated. The specific timing of each genetic event is crucial in tumor pathogenesis. These genetic events can be grouped into two broad categories. Eighty-five percent of tumors exhibit chromosomal instability but stability of microsatellite DNA; the typical genetic events associated with these tumors are illustrated in A. The remaining 15% of tumors are characterized by microsatellite instability; the key genetic events in these tumors are illustrated in B. Modified from Chung (2000).

1993; Winde *et al.*, 1993; Winde *et al.*, 1995). In addition, six months of twice-daily treatment with 400 mg of celecoxib, a selective cyclooxygenase-2 inhibitor, has been shown in a double-blind, placebo-controlled study to cause regression of polyps in patients with FAP (Steinbach *et al.*, 2000). The efficacy and safety of celecoxib and sulindac have not yet been directly compared. Any benefit by these agents is likely to be reversible, since an increase in the number and size of polyps has occurred in patients three months after sulindac is stopped (Giardiello *et al.*, 1993). So far, there is no sufficient information on the long-term benefits of chemoprevention. Also, the development of a new colorectal carcinoma in a patient with FAP who was receiving sulindac has been reported (Keller *et al.*, 1999). Therefore, these treatments should not now replace the treatment of surgical removal of the colon, a standard of care for patients with FAP.

Animal models of FAP have provided considerable insight into potential chemopreventive regimens (Giardiello *et al.*, 1993). In these mice, a variety of NSAIDs were found to inhibit tumor formation: aspirin (Barnes and Lee, 1998), piroxicam (Jacoby *et al.*, 1996) and R-flurbiprofen (Wechter *et al.*, 1997), and two metabolites of sulindac: sulindac sulfide and sulindac sulfone (Mahmoud *et al.*, 1998). Sulindac sulfide has active anti-inflammatory activity and ability to inhibit cyclooxygenase (Cox). Treatment with this metabolite in animals results in dramatic reductions in the number of polyps. Interestingly, a less potent but significant reduction in mouse tumors is found using sulindac sulfone, although it has no anti-inflammatory activity and no ability to inhibit Cox. This suggests that sulindac sulfone, and other drugs of this class, have additional neoplastic effects by increasing the degree of apoptosis.

Studies of patients with FAP prompted evaluation of use of aspirin and other NSAIDs in the general population. The Melbourne Colorectal Cancer Study was the first to show that regular aspirin use lowered risk of colorectal cancer (Kune *et al.*, 1988). A series of case-control studies demonstrated a 40 to 50 % decrease in the relative risk of this malignancy among continuous aspirin users (DuBois *et al.*, 1996).

Prospective studies were also used to assess aspirin and subsequent development of colorectal cancer or polyps in the general population. The initial cohort study (Paganini Hill *et al.*, 1989) showed no protective effect, however, all subsequent studies (at least 8) have shown protection with risk being reduced by at least 40% (Young *et al.*, 2000).

In a large US study (Thun *et al.*, 1992; Thun *et al.*, 1991), aspirin intake of more than 662,000 US adults was documented and the cohort was then followed-up for seven years. With infrequent aspirin use (less than once a month), the relative risks of death due to colon cancer were 0.77 for men and 0.73 for women; this risk decreased further to 0.60 for men and 0.58 for women in aspirin regular user (more than 16 times per month). A dose response relationship was noted. Of additional interest in this study was the finding that other gastrointestinal cancers were also reduced. The relative risk of gastric cancer was found to be 0.53 with frequent aspirin use, and the relative risk of oesophageal cancer was 0.59. Given the large size and prospective design of the study, it strongly supports the view that NSAIDs have a protective effect against a range of gastrointestinal malignancies.

In the Harvard Health Professionals Follow-up Study, a relative risk of colorectal cancer was 0.68 at first questionnaires in 47,900 male regular users of aspirin (at least twice per week) and decreased to 0.35 four or more years after (Giovannucci *et al.*, 1994). 551,000 nurses who documented aspirin usage were followed over a period of eight years in

the Nurses Health Study. Four years of using aspirin did not reduce the relative risk (relative risk, 1.06) compared to non-users, however, relative risk decreased to 0.56 over a 10-20 year period (Giovannucci *et al.*, 1995).

The US Physicians' Health Study was a prospective, randomised, clinical trial which assessed the benefit of aspirin (325mg every other day for 4.7 years) in the prevention of cardiovascular disease but also followed impact on cancer (Gann *et al.*, 1993). It failed to show a benefit from aspirin treatment but the findings are difficult to interpret because of the relatively brief duration of therapy, the low dose of aspirin, and the failure to exclude those with polyps or cancer at the start of the study. Given a probable presymptomatic phase for colorectal cancer of five to ten years, treatments of less than five years are unlikely to show benefit.

In summary, the current pool of epidemiological studies provides strong evidence that at a 40-50% reduction in colorectal cancer could be anticipated from regular aspirin or NSAID use (Young *et al.*, 2000). However, the impact of adverse events related to NSAIDs is not clear and there is no sufficient details regarding dosage, duration, age for starting treatment, and options regarding different NSAIDS. This latter requirement is important because, in the end, the appropriateness of NSAID chemoprevention will depend largely on the balance between toxicity and benefit.

As for HNPCC, to date there is no known interventional strategy that reduces its tumor risk. Likewise, there is no strategy that is known to reduce the incidence of MSI tumors, which constitute 15% of sporadic tumors (Boland *et al.*, 2000). Oral calcium supplementation has not shown effects in HNPCC (Cats *et al.*, 1995). An in vitro study has

suggested that aspirin may selectively inhibit the growth of cells with MSI; however, this has to be confirmed in a clinical trial in future (Ruschoff *et al.*, 1998).

Numerous non-NSAID agents have emerged as candidates to prevent colorectal carcinogenesis. Most of these agents have been found to exert a significant effect in animal models, including DMFO11, green tea extracts (Epigallocatechin gallate) (Weisburger *et al.*, 1998), curcumin (Kawamori *et al.*, 1999), folic acid (Giovannucci *et al.*, 1998; Kim *et al.*, 1996), mono- and dihydroxy vitamin D3 (Iseki *et al.*, 1999), selenium (Reddy *et al.*, 1997), dietary flavonoids (diosmin, hesperidin) (Tanaka *et al.*, 1997) and ursodeoxycholic acid (Earnest *et al.*, 1994). However, data from human trials are not yet available.

It will be apparent that there are multiple ways in which diet and chemopreventive agents may influence colorectal tumorigenesis. Although the mechanisms by which diet and chemopreventive agents prevent colorectal tumours remain unclear, one of the mechanisms thought to be important is induction of apoptosis in the tumours.

1.2.4 Dysregulation of apoptosis in colorectal cancer

Normal colonic crypts are composed of stem cells at the base, a proliferation and a differentiation zone in the lower third of the crypt, a migration zone in the upper two-thirds and the surface epithelium where senescent cells are eliminated by apoptosis. Apoptosis defined as a normal process of cell suicide is critical for development and tissue homeostasis. Colonic epithelial cells migrate from the base of the crypt to the surface epithelium in 6-7 days. The normal architecture of the crypt is maintained by a balance between cell proliferation at the base and apoptosis at the top of the crypt and surface epithelium.

Histopathologically, most sporadic colorectal cancers go through progressive changes called the adenoma-carcinoma sequence in which the balance between cell proliferation and apoptosis becomes progressively dysregulated. Bedi et al (Bedi *et al.*, 1995) examined colorectal epithelium from normal mucosa, adenomas from FAP, sporadic adenomas, and carcinomas. They found that the transformation of colorectal epithelium to carcinoma was associated with a progressive inhibition of apoptosis. Other studies indicated that apoptosis is higher in human colorectal adenomas than in carcinomas (Partik *et al.*, 1998; Tsujitani *et al.*, 1996). However, most studies on apoptosis of colonic epithelium have focused on the carcinoma stage of the cellular transformation (Sinicrope *et al.*, 1996). The inhibition of apoptosis in colorectal cancers may contribute to tumor growth, promote neoplastic progression, and confer resistance to cytotoxic anticancer agents.

The stem cells at the base of the crypt are susceptible to somatic mutations and these mutations persist during the migration along the crypt axis but will be eliminated by apoptosis at the surface epithelium. Therefore, normally, a large number of mutations will have no permanent consequences since apoptosis will eliminate these genetically altered cells. However, genes directly involved in the normal apoptotic process can themselves be mutated causing a dysregulation of apoptosis (Abdel Rahman *et al.*, 1999).

Dysregulation of apoptosis in the colonic epithelial cells might result from a mutation of genes encoding apoptotic proteins. This has been demonstrated in the case of Bax, a pro-apoptotic member of the Bcl-2 gene family (Abdel Rahman *et al.*, 1999). Mutations in Bax, rather than mutations in p53, may contribute to the adenoma-carcinoma transition in HNPCC tumorigenesis (Yagi *et al.*, 1998). Approximately 50% (21 out of 41) of human colon adenocarcinomas with microsatellite mutator phenotype were found to have

frameshift mutations in Bax. This suggests that inactivating Bax mutations are selected during the progression of colorectal tumors with microsatellite mutator phenotype and that the wild-type Bax gene plays a suppressor role in a p53-independent pathway for colorectal carcinogenesis (Rampino *et al.*, 1997). Furthermore, one study showed that low levels of Bax expression play a role in late stage colorectal cancer (Kanavaros *et al.*, 1999).

Another pro-apoptotic member of Bcl-2 family is Bak, which has been found to be down-regulated in a high proportion of colorectal tumours (Krajewska *et al.*, 1996). While in one report, both adenomas and carcinomas of the colon had increased levels of Bak compared to the normal colonic mucosa, but Bak expression was higher in adenomas correlating with increased apoptosis (Partik *et al.*, 1998). It is known that Bak plays an important role in regulation of intestinal cell apoptosis (Krajewski *et al.*, 1996; Moss *et al.*, 1996).

Bcl-2 is an important anti-apoptotic protein, which can even prevent caspase-independent cell death (Okuno *et al.*, 1998). It is possible that conditions of nutritional stress in colonic tumours may contribute to Bcl-2 upregulation (Singh and Paraskeva, 1998). Since many colorectal tumour have increased levels of Bcl-2 (Hague *et al.*, 1994), dysregulation of Bcl-2 may be important in colorectal carcinogenesis, especially in the early stage of the adenoma-carcinoma sequence (Yang *et al.*, 1999). One study showed that the positive immunohistochemical expression of Bcl-2 oncoprotein was associated with a significantly higher incidence of liver metastasis from colorectal cancer (Ishijima *et al.*, 1999). Bcl-2, however, fails to protect epithelial cells undergoing Bak-mediated apoptosis (Hague *et al.*, 1997).

Another anti-apoptotic member of the Bcl-2 gene family is Bcl-xL which is capable of protecting cells from p53-mediated apoptosis (Schott *et al.*, 1995). Elevated expression of Bcl-xL was found in colorectal tumours (Krajewska *et al.*, 1996; Maurer *et al.*, 1998); 83% of the cancers had increased Bcl-xL mRNA expression. The median Bcl-xL level was 3.4-fold higher in carcinomas than in normal tissue (Maurer *et al.*, 1998). In an animal model, elevated levels of bcl-xL protein may have considerable significance for azoxymethane-induced rat colonic adenocarcinomas as well as in human colorectal cancer (Hirose *et al.*, 1997).

Studies have also shown dysregulation and mutations in the caspase family in colorectal cancer, especially in caspases 3 and 5 (Leonardos *et al.*, 1999; Schwartz *et al.*, 1999). As a result, cells with mutations in the apoptotic machinery will accumulate at the top of the crypt and surface eptihelium and will be unable to detach and to die, contributing to neoplastic transformation.

Other genes indirectly involved in apoptosis are also commonly mutated. K-ras is mutated as early as the adenoma stage in more than 50% of adenomatous polyps. p53, which controls the elimination of altered cells through apoptosis, is mutated in 75% of colon cancers.

In summary, homeostasis of the normal colonic mucosa relies on a balance between proliferation at the base and apoptosis at the surface epithelium. The factors that govern the switch from regulated to unregulated apoptosis are therefore important to investigate further in the context of the pathobiology of colon cancer.

1.3 OVERVIEW OF APOPTOSIS

All multicellular organisms require a mechanism to counterbalance cell division in order to regulate cell number and remove unwanted cell types during development. In 1965, Lockshin and colleagues introduced the concept of "programmed cell death" to describe the phenomenon that had long been observed in embryogenesis where certain predetermined cells in the embryo would die at a particular stage during development (Lockshin and Williams, 1965). Later, Kerr et al (Kerr et al., 1972) linked this concept with a mode of cell death defined on strict morphological criteria, which they termed "apoptosis". This type of active cell death, which is under genetic control, is found in all tissues, including gastrointestinal epithelium, and can be induced by a wide variety of physiological stimuli. Apart from regulating cell number and type, apoptosis also performs an essential function of disposal of cells with damaged or mutant DNA. This prevents the proliferation of malignant clones and the propagation of cells containing viral DNA (Vaux, et al., 1994).

The similarity between the apoptosis-controlling genes in nematodes and mammals strongly suggests that a general pathway of apoptosis has been conserved during evolution. Although apoptosis occurs in diverse processes and cell types, the resultant morphological and biochemical changes are remarkably consistent, suggesting a fundamentally uniform mechanism.

1.3.1 The morphological and biochemical hallmarks of apoptosis

Apoptosis can be conveniently divided into two physiological stages. First is a "decision" phase. Following an appropriate stimulus, there is genetic control (such as regulation of CED9/Bcl-2 family) of whether or not a cell proceeds to death. Pro-apoptotic

stimuli will trigger activation of the apoptotic machinery. Secondly, there is an "execution" phase, as exemplified by CED-3/caspases, whose action contribute to the morphological and biochemical changes of apoptosis and lead to the death of the cell.

Apoptosis is characterised by its distinctive morphology (Kerr et al., 1972, 1994; Arends and Wyllie, 1991). For clarity, the sequence of morphological changes has been separated into three phases by Arends and Wyllie. First, a cell loses contact with the surrounding tissue and detaches from its substratum, the chromatin becomes condensed into crescent-like caps on the nuclear periphery, there is nucleolar disintegration, compaction of organelles with endoplasmic reticulum dilatation, clumping of ribosomal particles, cytoskeletal filament aggregation and cytoplasmic volume reduction. There is also loss of specialised surface structure, such as microvilli and junctional structures.

In the second phase, blebs of plasma membrane develop that can split away from the cells. This is a very dynamic process. Both the nucleus and cytoplasm split into fragments of various sizes, with the remaining cell becoming a round smooth membrane-bound body referred as an "apoptotic body". The apoptotic bodies are rapidly phagocytosed by neighbouring cells, including macrophages and parenchymal cells where they are degraded. If the fragmented cell is not phagocytosed, it will undergo secondary necrosis. There is progressive degeneration of the residual nuclear material and cytoplasmic structures. At this stage, the plasma membrane becomes permeable to vital dyes such as trypan blue. Apoptotic shrinkage, disassembly into apoptotic bodies and engulfment of individual cells characteristically occur without associated inflammation, which would be otherwise the consequence of releasing intracellular contents into tissues. Apoptosis is rapid, often completed in only 4-9 h.

DNA fragmentation is another hallmark of apoptosis. Degradation of DNA by endogenous DNases, which cut the internucleosomal regions into double-stranded DNA fragments of 180-200 base pairs (Wyllie, 1980), has been considered as a critical process in apoptosis. Internucleosomal fragmentation has been demonstrated with well-characterised apoptotic morphology in a wide variety of situations and cell types (Bortner *et al.*, 1995).

Amongst the caspase substrates are enzymes which regulate DNA structure, repair and replication (Nicholson and Thornberry, 1997). The DNase enzymes responsible for the fragmentation during apoptosis include DNA fragmentation factor (DFF40) (Liu *et al.*, 1998), caspase activated DNase (CAD) and in hematopoietic cells, NUC70 (Urbano *et al.*, 1998). DFF40 and CAD are present in normal cells as inactive heterodimers with the inhibitor proteins DFF45 (Liu *et al.*, 1997) and ICAD (inhibitor of CAD) (Sakahira *et al.*, 1998). These enzymes are selectively activated upon cleavage of the inhibitors by caspase-3 (Sakahira *et al.*, 1998; Liu *et al.*, 1997) or by other members of the caspase family (Tang and Kidd, 1998). Exposure of nuclei to activated CAD or DFF40 is sufficient to induce the nuclear morphologic changes typical of apoptosis (Enari *et al.*, 1998; Liu *et al.*, 1998). Formation of large size (50-300 kbp) DNA fragments precedes internucleosomal fragmentation, but the role of the above mentioned DNases in this process has not been studied (Bortner *et al.*, 1995).

Demonstration of the internucleosomal DNA fragmentation in apoptosis has been made by a variety of methods (Table 1.1). The DNA fragments are detectable as a ladder pattern in the electrophoresis of isolated DNA. With the terminal transferase mediated DNA nick end labelling (TUNEL) assay, cells containing DNA strand breaks become

Table 1.1 Common methods to assess apoptosis

Identification of morphological changes

Electron microscopy: loss of pseudopodia and microvilli, dilation of endoplasmic reticulum with morphological conservation of mitochodria (Wyllie et al., 1980)

Light microscopy: apoptotic bodies by staining cytospin preparations (or tissue sections) with hematoxylin or other stains

Fluorescence light microscopy: a mixture of acridine orange and ethidium bromide (Roger *et al.*, 1996), Hoechst 33342 stains (Morana *et al.*, 1996)

Identification of nuclear biochemical changes

DNA electrophoresis: a typical ladder pattern on agarose gel electrophoresis of DNA (Wyllie, 1980)

Fluorescent Hoechst 33258 dye/double-standed DNA assay (Teare *et al.*, 1997)

Flow cytometry: reduced DNA stainability, sub-G1 peak (Nicoletti *et al.*, 1991)

Isotope release: methyl[^{3}H] thymidine, [2-^{14}C] thymidine or 5-[^{125}I] iodo-2-deoxyuridine (Roger *et al.*, 1996)

In situ labeling of DNA strand breaks: TUNEL (TdT-mediated dUTP nick end-labeling) (Gavrieli *et al.*, 1992)

Decreased stability of condensed chromatin (Frankfurt *et al.*, 1996; Hotz *et al.*, 1992)

Identification of cytoplasmic apoptotic events

Disruption of mitochondrial transmembrane potential ($\Delta\Psi$m) (Zamzami *et al.*, 1995)

Transglutaminase induction (Fesus et al., 1996; Fesus et al., 1991)

Externalization of phosphatidylserine residues: 7-aminoactinomycin D, fluroresceinated annexin V stain (Fadok *et al.*, 1992; Vermes *et al.*, 1995)

DEVD-caspase activity assay (Liu *et al.*, 1996)

Immunoblotting assay of activated caspase (Liu *et al.*, 1996)

Immunoblotting assay of PARP cleavage (Gu et al., 1995; Lazebnik et al., 1994)

visible in light microscopic analysis (Gavrieli *et al.*, 1992). The quantification of apoptosis in tissue samples relies on this or related methods.

Different from apoptosis, necrosis refers to the morphology most often seen when cells die from severe and sudden injury, such as ischaemia, sustained hyperthermia, or physical or chemical trauma (Kerr et al., 1972, 1994). This is sometimes referred to as accidental cell death. In necrosis there are early changes in mitochondrial shape and function, and the cell rapidly becomes unable to maintain homeostasis. The plasma membrane may be the major site of damage; losing its ability to regulate osmotic pressure, the cell swells and ruptures. Loss of plasma membrane integrity and staining by vital dyes is an early, rather than late event. The contents of the cell are spilled into the surrounding tissue space and provoke an inflammatory response. Because cell injury of this type is often extensive, the inflammatory response is often desirable, so that debris may efficiently be cleared away and the process of repair begun.

Although the morphological and histological features of apoptosis and necrosis are very different, there may also be areas of overlap. Almost all stimuli inducing necrosis have been shown to also induce apoptosis in certain conditions. Following any sort of injury, if not capable of repair, the cell will attempt to initiate apoptosis so as not to trigger an inflammatory response. However, if the cellular injury is sufficiently great, the normal apoptotic pathways will be defeated and there will be early destruction of cellular membranes, leading to necrosis (Watson et al., 1995). This common basis for apoptosis and necrosis is supported by the observation that loss of mitochondrial potential is found early in both forms of cell death (Rosser and Gores, 1995; Petit et al., 1995).

1.3.2 Mechanisms of apoptosis

Apoptosis is genetically controlled, as demonstrated in the nematode *Caenorhabditis elegans.* Cell death occurring in the development of this worm involves the molecules CED-3 and CED-4, which are necessary for cell death, and CED-9, which protects cells from death. CED-9 acts as an inhibitor of CED-4 to prevent the death-inducing activity of CED-3 (Shaham and Horvitz, 1996). In mammals, CED-3 homologs are a family of at least 14 cysteine proteases with aspartate specificity, formerly called the ICE (interleukin-1β-converting enzyme) family, now called caspases (Alnemri, et al., 1996). The mammalian equivalent of the CED-4 molecule is Apaf-1 (apoptotic protease activating factor-1) (Zou, et al., 1997). CED-9 corresponds to the mammalian Bcl-2 gene (Hengartner and Horvitz, 1994).

1.3.2.1 Bcl-2 and Bax family

Bcl-2 was identified as a proto-oncogene in follicular B-cell lymphoma. In the lymphoma cells, Bcl-2 gene has been found at the breakpoint of the translocation between chromosome 18 and chromosome 14, where the gene is under the control of the immunoglobulin heavy chain intron enhancer (Tsujimoto *et al.*, 1985). This results in a transcriptional upregulation of the gene and overexpression of Bcl-2 protein. Bcl-2 has subsequently been shown to suppress most forms of apoptotic cell death as well as certain forms of necrotic cell death. Bax (Bcl-2 associated protein X) was the first pro-apoptotic member of the family to be identified. So far a large number of Bcl-2-related proteins, which possess at least one of four conserved motifs known as Bcl-2 homology domains (BH1 to BH4) have been isolated (Adams and Cory, 1998; Tsujimoto, 1998) and divided into three subfamilies.

1. Anti-apoptotic members such as Bcl-2, Bcl-xL, Bcl-w, Mcl-1, A1 (Bfl-1) and Boo, all of which have anti-cell death activity and contain BH1 to BH4, mostly, or at least BH1 and BH2.

2. Pro-apoptotic members such as Bax, Bak, Bad, Mtd (Bok), Diva, share sequence homology in BH1, BH2 and BH3 but not in BH4, although significant homology at BH4 has been noticed in some members.

3. `BH3-only proteins', these pro-apoptotic proteins include Bik, Bid, Bim, Hrk (DP5), Blk and Bnip3, Bnip3L, and share sequence homology only in BH3.

One feature of Bcl-2 family proteins is homo- and hetero-dimerization or heterodimerization between anti-apoptotic and pro-apoptotic proteins, which is considered to inhibit the biological activity of their partners (Oltvai *et al.*, 1993; Yang *et al.*, 1995). The BH1 and BH2 domains are required for Bcl-2 and Bcl-xL to interact with Bax and to suppress apoptosis (Borner *et al.*, 1994). In addition to the BH1 and BH2 domains, the BH4 domain is also required for anti-apoptotic activity (Huang *et al.*, 1998). In contrast, BH3 is essential and, itself, sufficient for pro-apoptotic activity (Chittenden *et al.*, 1995).

Bcl-2 family members have been shown to be both membrane-associated and cytosolic (Borner *et al.*, 1994). Bcl-2 is mainly located in endoplasmic reticulum membrane, inner (Hockenbery *et al.*, 1990) and outer membrane (Akao *et al.*, 1994) of mitochondria, and the nuclear envelope (Krajewski *et al.*, 1993). In contrast, Bax and Bcl-xL are found in the cytosol. Evidence shows that Bcl-2 family is involved in apoptosis by regulating mitochondrial functions, mainly in 1) mitochondrial permeability transition (MPT) pore opening, 2) release of apoptotic proteins from intermembrane space into the cytosol, such as cytochrome c (Kluck *et al.*, 1997; Liu *et al.*, 1996) and apoptosis-inducing

factor (AIF) (Susin *et al.*, 1999), which activates the downstream executional phase of apoptosis.

Cells overexpressing Bcl-xL fail to accumulate cytosolic cytochrome c or undergo apoptosis in response to genotoxic stress (Kharbanda, et al., 1997). It has been shown that Bcl-xL interacts with Apaf-1 and inhibits Apaf-1-dependent caspase-9 activation (Pan, et al., 1998; Hu, et al., 1998). Overexpression of Bax in stably transfected Jurkat cells induces the MPT, cytosolic accumulation of cytochrome c, caspase activation, cleavage of poly (ADP-ribose)-polymerase (PARP), DNA fragmentation, and cell death. Inhibition of the MPT prevents all manifestations of apoptosis, whereas caspase inhibition prevents PARP cleavage and DNA fragmentation but not cytochrome c release or cell death (Pastorino, et al., 1998)

1.3.2.2 Caspases

The other regulators of apoptosis are a unique family of cysteine-dependent protease called caspases. The members of this protease family share several common features (Earnshaw *et al.*, 1999). First, each caspase cleaves on the carboxyl side of aspartate residues. Secondly, each active caspase is a tetramer composed of two identical large subunits and two identical small subunits. Thirdly, each caspase is synthesised as a zymogen that contains an N-terminal prodomain, a large subunit and a small subunit. Finally, proteolytic cleavage, which forms the active caspase involves sequential cleavage at two or more aspartate residues, thereby separating the large and small subunits from one another and from the prodomain.

There are two classes of caspases, effector (or "downstream") caspases, which are responsible for most of the cleavages that disassemble the cell, and initiator (or

"upstream") caspases, which initiate the proteolytic cascade. Caspase-3, 6, 7 are the major effector caspases to date. Once activated, they are capable of cleaving the vast majority of polypeptides in apoptotic cells (Earnshaw *et al.*, 1999). Caspase-8 and –9 are the major initiator caspases identified. Zymogen forms of these enzymes have low but detectable protease activity (Earnshaw *et al.*, 1999). This activity increases when the prodomains of these zymogens interact with certain binding partners. Once activated, caspase-8 and –9 acquire the ability to cleave and activate the effector caspases.

1.3.2.3 Activation of caspase-9 and caspase-3

There are several pathways by which cells can be induced to apoptose. Mitochondrial proteins, such as cytochrome c (Lu, et al., 1997) and AIF (Susin, et al., 1996), have been identified as key signalling molecules of apoptosis. The mechanism of cytochrome c dependent activation of caspase-3 has been elucidated (Zou, et al, 1997; Li, et al., 1997). The N terminus of Apaf-1 has the CARD domain (caspase recruitment domain) (Hofmann, et al., 1997), which is shared by caspases -1, -2, -3, -4, -9 and CED-4. The C terminal side of the CARD domain is the CED homologue region, which contains Walker's A- and B-box consensus sequences. The rest of the C terminus has 12 WD-40 repeats. Two steps are required for activation of caspase-9: (1) binding of ATP or dATP to Walker's boxes, and (2) binding of cytochrome c to the WD repeats. These interactions allow exposure of the CARD domain. Upon binding cytochrome c, Apaf-1 becomes competent at binding pro-caspase-9, presumably because of cytochrome-c-induced conformational changes in the Apaf-1 protein (Li , et al., 1997). This results in proteolytic activation of pro-caspase-9 through an autoprocessing mechanism facilitated by the binding of pro-caspase-9 to the Apaf-1/cytochrome-c complex (Srinivasula, et al., 1998).

Once activated, caspase-9 can initiate a caspase cascade involving the downstream executioner caspase-3 (Zou, et al., 1997; Li, et al., 1997; Srinivasula SM, et al., 1998).

1.3.2.4 Activation of caspase-8

The death receptor mediated apoptosis pathway is important especially in the immune system (Boise & Thompson, 1996). Death receptors belong to the tumor necrosis factor (TNF) receptor gene family (Smith, et al., 1994) and contain a homologous cytoplasmic sequence termed the death domain (Tartglia, et al., 1993; Nagata, 1997). The best-characterized death receptors are CD95 and TNF-R (Smith, et al., 1994; Nagata, 1997). The ligands that activate these receptors are structurally related molecules that belong to the TNF gene superfamily (Smith, et al., 1994). Each CD95 ligand trimer binds three CD95 molecules (Smith, et al., 1994; Nagata, 1997). CD95 ligation leads to clustering of receptors via their death domains. Then, an adapter protein called FADD (Fas-associated death domain) binds through its own death domain to the death domains of the clustered receptors. FADD also contains a 'death effector domain' that binds to an analogous domain repeated in tandem within the zymogen form of caspase-8 (Boldin, et al., 1996; Muzio, et al., 1996). Upon recruitment by FADD, caspase-8 oligomerization drives its activation through self-cleavage (Muzio, et al., 1998).

1.3.3 Methods to assess apoptosis

It is important to discriminate between apoptosis and necrosis in pathological conditions. In most cases, none of the current laboratory techniques used alone allows for unambiguous identification of apoptotic cells. Most common methods are based on characteristic features of apoptosis in morphology, biochemistry, and plasma membrane changes. Table1.1 summarises some of the common methods, which are often used to

assess apoptosis. Generally, judgement of cell death in a given model always includes morphological examination coupled with at least one of the other assays. One reason is that an apoptotic cell rarely displays all of the known characteristic features; the use of several criteria is necessary to make a definite conclusion.

1.4 $P21^{WAF1/CIP1}$, CELL CYCLE AND APOPTOSIS

1.4.1 $p21^{Waf1/Cip1}$ and cell cycle

In the cell cycle, temporal order is imposed by the sequential activation of a series of serine/threonine protein kinases. Each functional kinase comprises a regulatory subunit, called a cyclin, and a catalytic subunit, called a cyclin dependent kinase (CDK). Cyclins D1, D2, and D3 together with their catalytic partners CDK4 and CDK6 appear to regulate events in the G_1 phase of cell cycle; the cyclin E-CDK2 complex operates at the initiation of S phase; cyclin A-CDK2 acts in S and G_2 phase and the cyclin B-CDK1 in M phase. Cyclin H-CDK7 on the other hand may act throughout the cycle by regulating the phosphorylation of other CDKs (Clarke, 1995).

There is a growing consensus that the retinoblastoma gene product (Rb) must play some part in the process. Rb is thought to function by negatively regulating the activity of transcription factors, such as E2F 1, 2 and 3, which in conjunction with other proteins (DP 1 etc) control the expression of specific genes essential for S phase (Peeper et al, 1994; Weinberg, 1995). Thus, S phase can only begin when sufficient E2F activity is released from the negative influence of Rb. In the normal course of events, this negative influence is overcome by phosphorylation. In G_0 and early G_1, Rb is hypophosphorylated and migrates as a single band in SDS-PAGE. As the cell progresses through G_1, Rb becomes

phosphorylated at multiple sites, culminating in a hyperphosphorylated state in late G_1 that is maintained throughout the remainder of the cycle (Peeper et al, 1994; Weinberg, 1995). It is clear that the cyclin D dependent kinases (or cyclin-CDK complexes) are responsible for initiating the phosphorylation of Rb. Very recently, it has been reported that Rb together with histone deacetylase negatively regulates the activity of transcription factors, such as E2F 1 (Alexander, et al., 1998; Magnaghi-Jaulin, et al., 1998).

There are two distinct categories of CDK inhibitors in mammalian cells (Elledge and Harper, 1994; Sherr and Roberts, 1995). The proteins designated $p21^{Waf1/Cip1}$ (p21), p27 and p57 appear to function as broad specificity inhibitors capable or interacting with complexes containing cyclins D, E and A. By contrast, members of the p15, p16, p18 and p19 family of proteins interact directly and specifically with CDK4 and CDK6 and therefore block the function of only the D type cyclins.

p21 is a poplypeptide of 164 amino acids with a CDK-binding site in the N-terminal portion and a proliferating cell nuclear antigen (PCNA) binding site in the C-terminal portion (O'Connor, 1997) (Fig 1.2). p21 is a potent inhibitor of most cyclin/CDK complexes and also inhibits the ability of PCNA to activate DNA polymerase δ, blocking polymerase δ dependent DNA replication (Waga *et al.*, 1994). In addition, p21 binding to PCNA regulates DNA mismatch repair (Umar *et al.*, 1996). p21 controls cell cycle progression through binding to G_1 cyclin/CDK complexes (Harper *et al.*, 1993; Harper *et al.*, 1995; Xiong *et al.*, 1993) and G_2 cyclin/CDK complexes (Medema *et al.*, 1998). DNA damage stimulates p21 transcription through p53-dependent mechanisms (Dulic *et al.*, 1994), whereas agents that regulate cellular differentiation may regulate p21 transcription through p53-independent mechanisms (Gartel and Tyner, 1999). Many of the studies

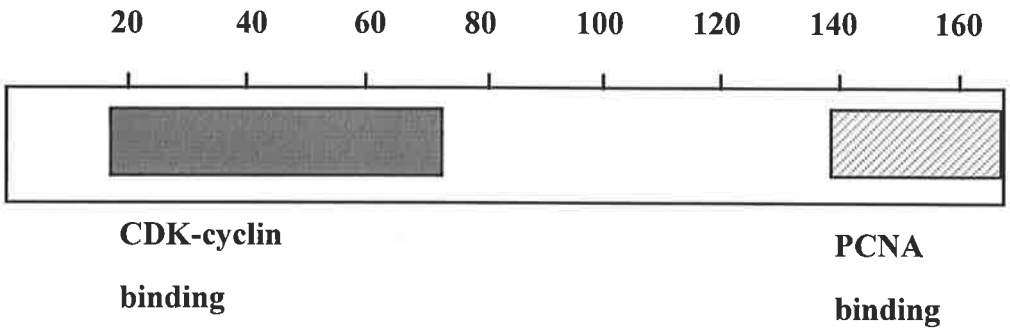


Figure 1.2 Structure of p21 showing the function of domains.

Large rectangle depicts the 164 amino acid p21 protein with the N-terminal CDK-binding site (filled rectangle) and the C-terminal PCNA-binding site (hatched rectangle).

reporting p53-independent regulation of p21 transcription demonstrate a requirement for GC-rich sites located within the first 100 bp of its promoter (Owen *et al.*, 1998; Prowse *et al.*, 1997). These sites have consistently been shown to bind members of the Sp family of transcription factors (Nakano *et al.*, 1997; Billon *et al.*, 1999; Li *et al.*, 1998; Moustakas and Kardassis, 1998; Sowa *et al.*, 1999; Kivinen *et al.*, 1999).

1.4.2 p21 and colorectal cancer

In normal colonic mucosa, p21 immunoreactivity was seen in the superficial third of the crypts (maturation compartment) and in surface (terminally differentiated) epithelium (Doglioni *et al.*, 1996; Sinicrope *et al.*, 1998). This may be consistent with its role in cell cycle arrest or differentiation.

In the progression from normal mucosa to colon adenocarcinoma, p21 expression was related inversely to proliferation and directly to terminal differentiation (Doglioni *et al.*, 1996). Loss of p21 protein expression accompanies progression of sporadic colorectal neoplasms but not HNPCC. In sporadic cases, a decrease in the frequency of p21 expression accompanied adenoma development and progression to carcinoma (Sinicrope *et al.*, 1998).

However, other studies suggest that p21 does not have a role in either onset or progression of most human cancers. Shiohara, et al (1997) analysed 471 primary samples from 15 types of human malignancies and 36 cell lines for structural alterations of the p21 gene. No changes were found in the coding region of p21 gene by polymerase-chain reaction-single-strand conformation polymorphism analysis. Other investigators showed that p21 knockout mice did not have an increased incidence of cancer.

1.4.3 p21 and apoptosis

Some studies have shown that p21 promotes apoptosis. For example, overexpression of p21 was found to be associated with giant cell formation and apoptosis in human breast carcinoma cell lines (Sheikh *et al.*, 1995). Inhibition of vascular smooth muscle cell growth by overexpression of human p21 gene was accompanied by induction of apoptosis through an inappropriate increase in bax protein. p21 promoted ceramide-induced apoptosis by enhancing the expression of Bax in human hepatocarcinoma cells.

However, most studies have shown that p21 is anti-apoptotic. For example, anti-sense oligonucleotides which block p21 expression facilitate, rather than suppress, apoptosis in neuroblastoma cells (Teare *et al.*, 1997). G_1 cell cycle arrest resulting from p21 can prevent apoptosis following UV-irradiation or treatment with an RNA polymerase II inhibitor, while serum starvation, which also synchronised cells in G_1 but did not induce p21, did not protect cells from apoptosis (Bissonnette & Hunting, 1998). It is possible that by maintaining the cells in G_1, p21 prevents critical events leading to caspase activation.

Data from multidrug resistant cell lines also demonstrated that p21 is involved in multidrug resistance, a common clinical phenomenon. ME-180 cervical carcinoma cells were selected for high or low apoptotic sensitivity to cis-diammedichloroplatinum. Cellular levels of p21 inversely correlated with cis-diammedichloroplatinum responsiveness with high levels of p21 expressed in drug-resistant ME-180 cells in the absence of elevated p53 (Donato et al, 2000). Zhang et al (1999) reported that A549/CPT cells, a multidrug-resistant cell line in which caspase-3-like protease activity was strongly attenuated, were resistant to anti-cancer drug-induced apoptosis, compared with parental A549 cells. Transfection of either Bcl-2 or p21 cDNA into parental A549 cells resulted in

resistance to apoptosis. Furthermore, the co-treatment of cells with anti-sense oligodeoxy-nucleotides to p21 and Bcl-2 restored drug susceptibility in A549/CPT cells more effectively than either one of them alone. These results indicate that co-induction of Bcl-2 and p21 in A549/CPT cells may be involved in acquired drug resistance by inhibiting caspase-mediated apoptosis.

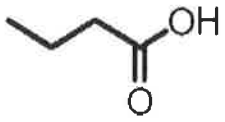
Furthermore, work from Vogelstein's laboratory (Polyak, et al., 1996) showed that expression of p53 in a series of colorectal cancer cell lines yielded growth arrest in some lines and apoptosis in others. Inactivation of p21 by homologous recombination converted cell growth to apoptosis, suggesting p21-mediated growth arrest can protect cells from apoptosis. p21 antisense therapy radiosensitizes human colon cancer by converting growth arrest to apoptosis (Tian *et al.*, 2000).

One possible mechanism by which p21 suppresses apoptosis is by binding to procaspase-3 to form a complex, which prevents activation of caspase-3 and results in resistance to Fas-mediated apoptosis (Suzuki et al, 1998).

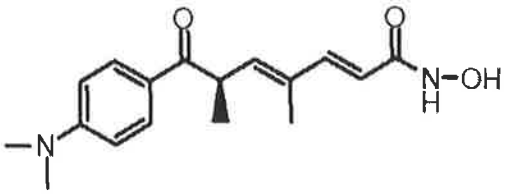
1.5 BUTYRATE AND CELLULAR BIOLOGY

1.5.1 Production, structure and metabolism of butyrate

The bacterial fermentation of fibre in the colon produces methane, carbon dioxide, water as well as major products, short chain fatty acids (butyrate, acetate and propionate). Butyric acid is a 4-carbon fatty acid (Fig 1.3). Miller and Wolin (1979) compared colonic fermentation by various bacteria in the rumen and human faeces and developed a fermentation equation based on the assumption that other minor products can be derived from the major short chain fatty acids metabolites as:



Butyrate



TSA

Figure 1.3 The structures of butyrate and TSA.

34.5 $C_6 H_{12}O_6 \rightarrow$ 43 Acetate + 11 Propionate + 5 Butyrate +23.75 CH_4 + 34.25 CO_2 + 10.5 H_2O

Short chain fatty acid concentration in the content of the human large intestine is approximately to be 75 mM Acetate +30 mM Propionate + 20 mM Butyrate. The various biochemical pathways and fermentation products leading to production of butyrate by species of colonic bacteria is described by Miller and Wolin (1979).

Colonic epithelium can metabolise these short chain fatty acids to produce ketones, CO_2 and energy, and there is evidence that, in colonic epithelium, butyrate may be the preferred energy source over glucose or glutamine (Roediger, 1982). 70% of the total energy consumption of the colonocyte may come from butyrate (Scheppach, 1994). In addition, those short chain fatty acids derived from bacterial fermentation of dietary fibre that are not utilised by the human colon are absorbed into the bloodstream. The majority of acetate passes through the liver and can be recovered in peripheral blood, whereas butyrate and propionic acid remain in the liver.

1.5.2 Modulation of chromatin structure and transcription from specific genes

Butyrate is able to alter the expression of many genes. The effects of butyrate on gene expression have been traced to alterations in the structure of nuclear chromatin. In vivo, the core histones, such as H3, H4, H2A, and H2B form a tetramer $(H3H4)_2$ and a pair of dimers (H2A.H2B) form a core around which the DNA wraps. Eukaryotic chromatin at low salt concentrations appears, under the electron microscope, as successive histone-DNA beads that are interspersed by a continuous DNA thread, commonly described as beads-on a-string. As the salt concentration is increased and a fifth histone, H1 (the most variable), is introduced, the thread twists and compacts to form a regular

fibre measured to be typically 30 nm in diameter (Bradbury, 1992). This structure is compacted even further in vivo. In vivo and in vitro, the histones interact with each other as well as with the DNA. Specific acetylation levels of histone H4 have been associated with replication (Woodland, 1979) and transcription (Waterborg, 1984). Highly reversible acetylated histone forms were produced after addition of low levels of butyrate to cultured cells (Riggs, et al., 1977). This is due to inhibition of deacetylase by butyrate (Candido, et al., 1978; Sealy & Chalkley, 1978; Vidali, et al., 1978). The deacetylase is responsible for removing acetyl groups, and once inhibited, hyperacetylation of the histones occurs, which can loosen the local chromatin structure and increase the accessibility of transcriptional regulatory proteins to specific DNA sequences (Lee *et al.*, 1993; Vettese Dadey *et al.*, 1996). In this way, butyrate influences a number of genes (Boffa *et al.*, 1994), either turning a repressed gene on or inactivating a gene engaged in transcription. Therefore, it is possible that some death genes may be activated and apoptosis could be triggered in butyrate-treated colorectal cancer cells. That histone acetylation triggers apoptosis by butyrate is supported by the finding that trichostatin A (Fig 1.3), a more specific inhibitor of histone deacetylase, mimics butyrate in the kinetics of induction of apoptosis in colon cancer cells (Medina *et al.*, 1997).

The butyrate induced hyperacetylation of H3 and H4 is also accompanied by increased incorporation of ADP-ribose (Golderer & Grobner, 1991), and DNA synthesis appears to be enhanced in hyperacetylated nucleosomes (Ramanathan & Smerdon, 1989). Poly(ADP)-ribose polymerase functions as a histone-shuttle mechanism, releasing DNA from the nucleosome and facilitating DNA excision repair, a process known to stimulate the processing of ADP-ribose (Realini & Althaus, 1992).

In addition, butyrate inhibits phosphorylation of histones H1 and H2A in HeLa cell (Boffa, et al., 1981). A correlation between increased histone acetylation and increased H3 phosphorylation was found when HeLa cells were cultured for 16 hours in the presence of sodium butyrate (Whitlock, et al., 1983).

1.5.3 Cell Cycle Arrest

When treated with millimolar concentrations of butyrate, many cell types undergo growth arrest in both the G_1 phase of cell cycle (Wintersberger *et al,* 1983; Darzynkiewicz *et al*, 1980; D'Anna *et al*, 1980; Barnard and Warwick, 1993) and at G_2/M transition (Gupta *et al.*, 1994; Heerdt *et al.*, 1997; Yamada *et al.*, 1992). However, the molecular mechanisms of these G_1 and G_2 arrests have not been elucidated. Several mechanisms have been proposed. Firstly, histone phosphorylation is closely associated with progression through the cell cycle. Dephosphorylated H1 was correlated with G_1 arrest in butyrate treated Chinese hamster cells (D'Anna *et al.*, 1980). Secondly, increased histone acetylation induced by butyrate was associated with the G_1-S arrest (Kruh, 1982). Finally, butyrate can also cause dephosphorylation of the Rb protein in mouse fibroblasts (Buquet Fagot *et al.*, 1996). Some experiments have shown that the hypophosphorylated Rb induced by butyrate is associated with arrest in G_1 (Vaziri *et al.*, 1998; Yen and Sturgill, 1998) and G_2 (Yen and Sturgill, 1998).

1.5.4 Butyrate and p21

The induction of p21 by butyrate has been reported in colorectal cancer cell lines (Siavoshian *et al.*, 1997; Siavoshian *et al.*, 2000; Archer *et al.*, 1998; Heerdt *et al.*, 1998;

Nakano *et al.*, 1997), and in other types of cell such as hepatocellular carcinoma cells (Yamamoto *et al.*, 1998), normal C3H10T1/2 cells (Janson *et al.*, 1997), NIH3T3 cells (Xiao *et al.*, 1997) and prostate epithelial cancer cells (Huang *et al.*, 1999). Up-regulation of p21 by butyrate mediates growth arrest (Archer *et al.*, 1998) and differentiation of colorectal cancer cells (Siavoshian *et al.*, 2000; Litvak *et al.*, 1998). However, the action of p21 in butyrate-induced apoptosis is not clear.

The mechanism by which butyrate up-regulates p21 expression, is likely to be a p53-dependent response to some stimuli eg DNA-damaging agents or it may be a p53-independent pathway. It has been reported that butyrate decreased p53 expression and increased p21 expression in hepatoma HCC-T and HCC-M cells, which have the wild-type p53 gene (Saito *et al.*, 1998). Further induction of p21 by butyrate may be a direct effect mediated by a butyrate-responsive element in the promoter region of the p21 gene (Nakano *et al.*, 1997). During this direct pathway, transcription factor ZBP-89, cooperating with histone acetyltransferase p300, also plays a critical role in butyrate activation of the p21 promoter (Bai and Merchant, 2000).

1.5.5 Differentiation

Butyrate can induce markers of differentiation at the same time as inducing cell growth arrest in tumor cell lines. Incubated with butyrate, tumor cell lines assume a phenotype more like the original non-neoplastic tissue. Butyrate at 1 mM increases 6-fold alkaline phosphatase activity (Whitehead, et al., 1986). Other markers of differentiation induced by butyrate include brush-border hydrolases, diaminopeptidylpeptidase (Chung, et al., 1985), carcinoembryonic antigen (Niles, et al., 1988; Saini, et al., 1990; Gibson, et al., 1992) and membrane-associated glycoproteins and glycolipids (Siddiqui, et al., 1984).

1.5.6 Butyrate-induced Apoptosis

More recently, butyrate has been shown to induce several colon cancer cell lines to undergo apoptosis at concentrations of 1-5 mM, well within the physiological range. (Hague, et al., 1993, 1995). This effect was also confirmed in the human myeloid HL-60 cell line (Calabresse, et al., 1993) and in the Burkitt's lymphoma BL-30 cell line (Filippovich et al., 1994). Butyrate induced apoptosis in 7 adenoma or carcinoma cell lines, however, the response of these cell lines was different. 2 of the 4 carcinoma cell lines were only partially sensitive (Hague *et al.*, 1995). Although it has been known that butyrate induces growth arrest, differentiation and apoptosis of tumour cells, the relationship between them is unclear. It is not clear whether growth arrest precedes differentiation and apoptosis or whether growth arrest results in separate pathways of differentiation or apoptosis. Furthermore, little is known about the molecular mechanism of butyrate-induced apoptosis though butyrate-induced apoptosis involves activation of caspase-3 (and/or related caspases) (Medina, et al., 1997).

In MCF-7 cells, the butyrate-induced apoptosis was closely associated with the down-regulation of expression of Bcl-2 mRNA and Bcl-2 protein. Stable overexpression of Bcl-2 resulted in protection of MCF-7 cells from growth-inhibitory effects of butyrate (15% growth inhibition compared to 60% growth inhibition in the parental cells) and from butyrate-induced stimulation of apoptosis (5-fold increase in apoptosis compared to 27-fold in parental MCF-7 cells) (Mandal & Kumar,1996). In colorectal carcinoma, butyrate-induced apoptosis of DiFi cells was accompanied by inhibition of expression of Bcl-2 and this cellular effect of butyrate was inhibited by Bcl-2 overexpression (Mandal, Wu and Kumar, 1997). In human colonic adenoma cells, expression of constitutively high levels of

Bcl-2 only conferred protection against apoptosis when bak levels were not elevated in response to butyrate, and expression of constitutively high levels of Bcl-2 did not counter the effects of bak (Hague, et al., 1997). However, butyrate also increased the Bcl-2 level in a human eosinophilic leukemia cell line, EoL-1 (Tai, et al., 1996) and in human hepatoma (HCC-T and HCC-M cell lines) cells (Saito, et al., 1998).

Moreover, butyrate not only induces p53-independent apoptosis (Hague *et al.*, 1993; Janson *et al.*, 1997) but partially inhibits p53-mediated apoptosis as well. As to the latter effect, low concentrations of butyrate were more effective than higher concentrations (Janson *et al.*, 1997). In support of this, butyrate is able to down-regulate wild-type p53 (Gope and Gope, 1993; Palmer *et al.*, 1997) and mutant p53 (Palmer *et al.*, 1997) in colonic tumour cells. Therefore, lumenal factors are able to affect directly the expression of p53 protein in colonic epithelial cells (Palmer *et al.*, 1997).

In addition, butyrate treatment can also cause a reversal in the phosphorylation status of the pRb in colon tumor cell line HT29 (Gope and Gope, 1993).

1.5.7 Trichostatin A

Another inhibitor of histone deacetylase, trichostatin A (TSA), is also able to induce cell growth arrest, differentiation and apoptosis in a wide variety of tumour cells such as neuroblastoma, melanoma, and leukemia cells, as well as cells from breast, prostate, lung, ovary, and colon cancers (Marks *et al.*, 2000). Although both butyrate and TSA inhibit histone deacetylase, their structures are quite different. Fig 1.3 shows their structure. Butyrate acts in millimolar concentrations while TSA works in nanomolar concentrations (Marks *et al.*, 2000).

1.6 SULINDAC AND ITS CELLULAR EFFECTS

1.6.1 Sulindac and its derivatives sulindac sufide and sulfone

Sulindac is a classic nonsteroidal anti-inflammatory drug known to inhibit chemical carcinogenesis in rodent models and cause regression of adenomas in patients with adenomatous polyposis coli (Waddell and Loughry, 1983). Sulindac is a prodrug that is metabolized to a pharmacologically active sulfide derivative, which potently inhibits prostaglandin synthesis, and a sulfone derivative, which essentially lacks prostaglandin synthesis inhibitory activity (Piazza *et al.*, 1995). Both sulindac sulfide and the sulfone inhibit the growth of cultured tumor cells, although the cellular mechanism(s) responsible for the anti-neoplastic activity of sulindac derivatives is unknown. Neither sulindac sulfide nor the sulfone induced differentiation of HT-29 cells, but both drugs strongly induced apoptosis (Piazza *et al.*, 1995)

1.6.2 Cyclooxygenase-2 and colorectal cancer

There are two known isoforms of Cox. Cox-1 is constitutively expressed in most tissues and has a role in tissue homeostasis. It is largely responsible for protecting upper gastrointestinal mucosa from injury. Its inhibition is associated with a substantial risk of gastric and duodenal erosions and ulcers. Cox-2 is inducible by a variety of cytokines and mitogens and can not be detected in most tissues under physiological conditions (Smalley and DuBois 1997, Sano, Kawahito et al. 1995). More importantly, Cox-2 is not ordinarily expressed in colorectal epithelium but is highly expressed in many neoplastic tissues, beginning with the earliest adenomatous polyps. In contrast, Cox-1 expression is not

increased in neoplastic tissues, compared with normal tissue (Eberhart *et al.*, 1994; Sano *et al.*, 1995).

Several pieces of evidence implicate Cox-2 in colorectal tumorigenesis. Expression of Cox-2 is elevated in colorectal tumors (Eberhart *et al.*, 1994; Sano, Kawahito et al. 1995; Kutchera *et al.*, 1996). In some laboratory models, Cox-2 inhibition can halt the growth of cultured colon cancer cells. In the $APC^{\Delta 716}$ knockout mouse model, where there is development of a large number of intestinal polyps, a null mutation in Cox-2 dramatically reduced the number and size of the intestinal polyps (Oshima, Dinchuk et al. 1996). Rat intestinal epithelial (RIE) cells, which over-express Cox-2, were resistant to butyrate-induced apoptosis (Tsujii and DuBois 1995). All these findings suggest that high levels of Cox-2 may contribute to colorectal carcinogenesis.

1.6.3 Mechanism of antitumour activity of sulindac

Initially, NSAIDs were found to be able to inhibit cell proliferation and cause cell cycle arrest (Adolphe *et al.*, 1972; Hial *et al.*, 1977; Bayer and Beaven 1979; Bayer *et al.*, 1979). More recently, NSAIDs were shown to be capable of inducing apoptosis in cultured cell lines (Piazza *et al.*, 1995; Shiff *et al.*, 1995; Lu *et al.*, 1995; Tsujii and DuBois 1995). The molecular basis of these effects has been attributed to inhibition of Cox and the resulting decrease of prostaglandin production (Marnett, 1992).

It has been proposed that colorectal tumorigenesis is promoted by over-expression of Cox-2 because of enhanced prostaglandin formation (Boolbol *et al.*, 1996). However, some evidence argues against the hypothesis that the anti-neoplastic effects of NSAIDs are by inhibition of production of prostaglandins. NSAIDs inhibit the proliferation and induce of apoptosis in colon cancer cells that lack Cox transcripts and do not produce

prostaglandins even when exogenously stimulated (Hanif *et al.*, 1996). Sulindac sulfone does not inhibit Cox ; however, it still has antineoplastic ability (Piazza *et al.*, 1995; Piazza *et al.,* 1997). Prostaglandins cannot rescue NSAID-induced growth arrest and apoptosis (Hanif *et al.*, 1996; Narisawa *et al.*, 1984; Chan *et al.*, 1998). In transformed embryonic fibroblast cells with knockouts of both Cox-1 and Cox-2, NSAIDs are still antiproliferative as well as inducing apoptosis (Zhang *et al.,* 1999).

Since there may be a prostaglandin-independent mechanism for the anti-tumor effect of NSAIDs and a vital role for Cox-2 in colorectal carcinogenesis, tumor suppressive effects of NSAIDs might be due to an increase of the prostaglandin precursor arachidonic acid. In support of this hypothesis, treatment of colorectal cancer SW480 and HCT116 cells with NSAIDs resulted in a dramatic increase in arachidonic acid which in turn stimulated the conversion of sphingomyelin to ceramide, a known mediator of apoptosis (Chan *et al.*, 1998).

Very recently, it was reported (He *et al.*, 1999) that sulindac sulfide and sulfone inhibit the activity of PPARδ. PPARδ expression was elevated and was identified as a target repressed by APC in colorectal cancer cells. This repression was mediated by β-catenin/Tcf-4-responsive elements in the PPARδ promotor. Sulindac was able to disrupt the ability of PPARδ to bind its recognition DNA sequences. PPARδ is a transcription factor, belonging to the same class of steroid/thyroid hormone nuclear receptor family (Kastner *et al.*, 1995; Mangelsdorf *et al.*, 1995). The tumour suppressor APC, which is often mutated in familial adenomatous polyposis, also down regulates the transcriptional activity of PPARδ (He *et al.*, 1999) . It may be that one or more of the genes regulated by PPARδ is a gene whose product suppresses apoptosis in colorectal cancer cells.

1.6.4 Lipoxygenase and colorectal cancer

Arachidonic acid is metabolised through three pathways (Smith 1989). The first pathway produces the prostanoids (thromboxanes and prostaglandins) via the enzyme Cox. The second produces the leukotrienes via the enzyme lipoxygenase (Fig 1.4), and the third pathway produces the epoxyeicosatrienoic acids by P-450 epoxygenases.

Mounting evidence suggests that lipoxygenase products, leukotrienes and hydroxyeicosatetraenoic acids (HETEs), also exert profound biological effects on the development and progression of human cancer. 12- lipoxygenase mRNA expression has been well documented in many types of solid tumour cells, including those of prostate, colon, and epidermoid carcinoma cells (Chen *et al.*, 1994; Honn *et al.*, 1994). Also, 12-HETE production by some tumour cells, including prostate cells, has been positively correlated to their metastatic potential (Gao *et al.*, 1995; Tang and Honn, 1994). It has been shown that 12-HETE is a critical intracellular signalling molecule, stimulating PKC and eliciting the biological actions of many growth factors and cytokines that regulate transcription factor activation and induction of oncogenes or other gene products needed for neoplastic cell growth (Liu *et al.*, 1994; Liu *et al.*, 1991; Timar *et al.*, 1996). Additionally, PKC activation by 12-HETE mediates the release and secretion of cathepsin B, a cysteine protease involved in tumour metastasis and invasion, particularly in colon cancer cells (Honn *et al.*, 1994). Other lipoxygenase metabolites, particularly the 5-lipoxygenase products (5-HETEs), have been implicated in cancer development. 5-HETE directly stimulates prostate cancer cell growth (Ghosh and Myers, 1997). Arachidonate lipoxygenases are essential as regulators of cell survival and apoptosis in W256 (Tang *et al.*, 1996). It has been reported that colorectal cancer cell lines (HT-29 and HCT-15) were

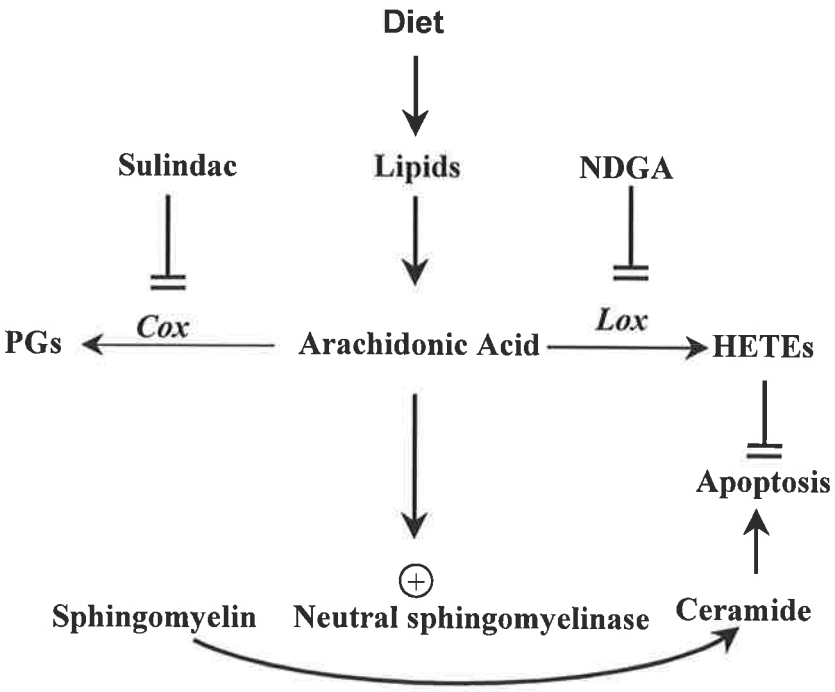


Figure 1.4 AA is generated by cytosolic and secreted phospholipase A2, which hydrolyzes plasma membrane lipids or lipids derived from the diet. AA is normally used as a substrate by the Coxs to produce eicosanoids such as prostaglandins. NSAIDs and NDGA inhibit the activity of the Coxs and Loxs, respectively, which increases the cellular pool of AA. AA stimulates neutral sphingomyelinase activity, which catalyzes the hydrolysis of sphingomyelin to generate ceramide. Ceramide acts as second messenger to activate the cellular apoptotic machinery.

stimulated in a time-and concentration-dependent way to proliferate by leukotriene B4 and 12-HETE, a p450-derived product (Bortuzzo *et al.*, 1996). In the same study, a competitive LTB4 antagonist inhibited LTB4-induced growth stimulation in HT-29 cells. Similar inhibition occurred in murine colon adenocarcinoma cells by 5-lipoxygenase inhibitors such as BWA4C and BWB70C at micromolar concentrations (Hussey and Tisdale, 1996). Additionally, LTB4 and LTA4 were found to be synthesised in human HT-29 (Cortese *et al.*, 1995; Sjolander *et al.*, 1993) and Caco-2 cells (Dias *et al.*, 1992). Therefore, all these data suggested that lipoxygenase product may be involved in colon carcinogenesis. Of intereste, butyrate was able to induce the expression of lipoxygenase (Kamitani *et al.*, 1998).

1.7 ZINC, APOPTOSIS AND COLORECTAL CANCER

1.7.1 Zinc and apoptosis

Zinc is an important trace element. It has been shown to been a structural and/or functional component of more than 200 metalloenzymes, as well as numerous zinc finger transcription factors. One important function of zinc is that it can reversibly protect cells from oxidation (Da Silva and Williams, 1991). These antioxidant properties were probably important in the context of regulation of apoptosis. From this viewpoint it is interesting that zinc shares features in common with the anti-apoptotic protein Bcl-2 which also has anti-oxidant properties (Korsmeyer *et al.*, 1993). Suppression of premature apoptosis may be a critical physiological function of zinc. In vivo, zinc is mainly present in two ways, 1) tightly bound pools within zinc proteins, which constitute a largely fixed pool of cellular zinc (associated with metalloenzymes and zinc finger proteins); 2) labile zinc pools, which are

loosely associated with proteins, lipids, cytoskeletal processes or sequestered in vesicles. These labile pools, which are believed to be important in cytoprotection and the regulation of apoptosis, are readily influenced by zinc deprivation or supplementation (Vallee and Falchuk, 1993; Zalewski *et al.*, 1993).

Elmes (1977) firstly proposed that zinc may be important in the regulation of apoptosis in 1977, observing increased frequencies of apoptotic cells in the small intestinal crypts of zinc-deficient rats. She attributed this apoptosis to a failure of DNA synthesis in these cells. Subsequent studies showed that the frequency of apoptotic cells was also markedly increased in many tissues of adult animals and in the neuroepithelium of foetal rats bone by zinc-deficient dams (Zalewski and Forbes, 1993). However, the extent to which apoptosis occurs in zinc-deficient humans is less clear. Increased apoptosis in zinc-deficient humans has been found in some pathological conditions, such as in peripheral leucocytes of patients with Down syndrome and in the severe T cell depletion of zinc malabsorption syndromes (Solomons, 1988). Zinc deficiency results not only in damage to the epithelial tissues including lesions in the skin and gastrointestinal epithelium (Solomons, 1988), but also in a heightened susceptibility of the epithelium to damage by other toxins (e.g. in the duodenum by colchicine (Dinsdale and Williams, 1977)). One explanation for these pathological changes is that there is increased apoptotic death of epithelial cells (Zalewski and Forbes, 1993). However, zinc deficiency can also lead to cell death by necrosis, especially when the apoptotic pathway is dysfunctional (Kolenko *et al.*, 1999).

It is not clear whether increased apoptosis in vivo is due to a direct consequence of a decrease in intracellular zinc in the affected cells or secondary to changes in other tissues or

both. Apoptosis in the thymus of zinc-deficient rodents was at least in part due to excessive levels of circulating glucocorticoids triggered by a zinc deficiency-associated stress response (Fraker and Telford, 1997). However, evidence from in vitro studies indicates that apoptosis can result directly from a decrease in intracellular zinc induced by culture of cells in either zinc-free medium or in the presence of membrane-permeant zinc chelators, such as TPEN.

The mechanisms by which zinc deficiency result in enhanced apoptosis remain unclear. First, earlier studies reported that zinc suppressed the endonucleases. For example, at a relatively low concentration (50 μM), zinc strongly inhibited Ca-induced DNA fragmentation in isolated nuclei (Cohen and Duke, 1984). Secondly, recent experiments showed that zinc is a regulator of caspase activation (Chai *et al.*, 1999). In support of this hypothesis, zinc blocked the conversion of an inactive caspase-3 zymogen precursor to an active form, rather than suppressing the activity of caspase-3 in cleaving its cellular substrates (Takahashi *et al.*, 1996). A similar finding has been recently reported in a study using Western blotting to track caspase-3 processing of HL-60 cells (Aiuchi *et al.*, 1998).

One possible target of zinc is caspase-6, which is known to cleave and activate the proenzyme form of caspase-3 (Liu *et al.*, 1996). Studies show that caspase-6 is highly sensitive to zinc (Stennicke and Salvesen, 1997; Takahashi *et al.*, 1996) and is suppressed at micromolar concentrations of zinc (Stennicke and Salvesen, 1997). The other possible target may be an initiator caspase, caspase-9 (Thornberry and Lazebnik, 1998).

Another recent study has shown the regulation of the anti-apoptotic Bcl-2-like and pro-apoptotic Bax-like mitochondrial membrane proteins by zinc (Fukamachi *et al.*,

1998). In zinc supplemented U937 cells, a significant increase in the ratio of Bcl-2/Bax was found (Fukamachi *et al.*, 1998). A high ratio of Bcl-2/Bax is thought to increase cellular resistance to apoptosis (Korsmeyer *et al.*, 1993). Other potential targets of zinc are the microtubular cytoskeleton, which is stablised by zinc and disrupted in apoptosis (Zalewski and Forbes, 1993), and the cytoplasmic glucocorticoid receptor in thymocytes (Fraker and Telford, 1997). In addition, zinc may also influence events at the level of gene expression, because zinc deprivation- induced apoptosis of neuron is suppressed by cycloheximide (Ahn *et al.*, 1998). It has been shown that zinc deficiency up-regulated expression of 30 genes while down-regulating expression of 17 genes (Blanchard and Cousins, 1996). One or more of these genes may be involved in apoptosis.

1.7.2 Zinc and colorectal cancer

Some studies have suggested that zinc metabolism may be abnormal in colorectal cancer. There was a positive correlation between zinc level and superoxide dismutase activity in colorectal adenoma and cancer (Magalova *et al.*, 1999). Others reported that Zn-superoxide dismutase content was similar between colorectal adenomas, carcinomas, and the corresponding normal mucosa (Janssen *et al.*, 1999; Janssen *et al.*, 1998). Urinary zinc output is increased in colorectal cancer (Hronek *et al.*, 2000; Melichar *et al.*, 1994) although the mean serum zinc levels were lowered only in advanced (Dukes stages C and D) colorectal cancer (Gupta *et al.*, 1993). Importantly, the levels of zinc were increased in human colon cancers compared with adjacent normal tissue (Gupta *et al.*, 1993; Song *et al.*, 1993). This is controversial since one other study has reported that zinc is reduced in colorectal cancer cells compared with normal tissue (Xiao and Henderson, 1992). Further studies by Song (Song *et al.*, 1995) suggested that zinc is required for up-regulation of the

inducible form of prostaglandin synthetase, and that maintaining an optimal zinc nutriture is important for normal prostaglandin synthesis in colonocytes. Since prostaglandin E_2 significantly increased the rate of zinc uptake of colonic tumour cells (Song *et al.*, 1995), abnormalities in prostaglandin E_2 synthesis in these cells may result in an abnormal zinc status.

1.8 PHYTIC ACID, ZINC AND COLORECTAL CANCER

1.8.1 Phytic acid and zinc

Myo-inositol hexaphosphate is present in plants, particularly in cereals and legumes, in concentrations ranging from 0.4 to 6.4% and is referred to as phytic acid. Phytate in food is composed of a mixture of different phosphorylated forms of inositol phosphate (Sandberg and Ahderinne, 1986); the hexaphosphate is usually the major form, but pentaphosphates, tetraphosphates and triphosphates are also present. In mammalian cells, inositol hexaphosphate and lower inositol phosphates ($InsP_{1\text{-}5}$) are present as intracellular molecules. Inositol $_{1,4,5}$-trisphosphate ($Ins(_{1,4,5})P_3$) plays a second messenger role via mobilising intracellular Ca^{2+} and regulating cellular functions including mitosis (Hirata and Murad, 1994). Inositol $_{1,3,4,5}$-tetrakisphosphate ($InsP_4$) has also been shown to induce Ca^{2+} sequestration. Higher forms of InsP, inositol $_{1,3,4,5,6}$-pentaphosphate ($InsP_5$) and inositol hexaphosphate ($InsP_6$) are also enriched, being the main components of InsP in mammalian cells; their concentrations range between 10 μM and 1 mM, much higher than any other InsP (Shamsuddin *et al.*, 1997).

It was shown in animal studies that phytate has an inhibitory effect on zinc absorption (O'Dell, 1969; Vohra *et al.*, 1965). The phosphate groups in inositol

hexaphosphate can form strong and insoluble complexes with cations such as zinc, and because the gastrointestinal tract of higher species lack any significant phytase activity, phytate-bound minerals will be excreted in the stool. The finding of zinc deficiency in human subjects in the Middle East led to the realisation that phytate can affect zinc status in humans as well (Halsted et al. 1972), and subsequent studies further confirmed this. Because staple foods in most part of the world contain phytate (e.g., corn, cereals, rice and legumes), it is obvious that zinc status may be compromised in significant proportions of the population. By using radioisotopes and whole-body counting, Sandström and colleagues found very low zinc bioavailability from soy-based infant formula compared with milk formula and human milk (Sandström et al. 1983). Moreover, the addition of phytate to milk formula reduced zinc absorption to a level similar to that from milk formula, supporting the hypothesis that the low zinc absorption from soy was due to its phytate content. Removal of phytate from soy protein by a precipitation process increased zinc absorption significantly (Lönnerdal et al. 1988). An experiment in suckling rat pups using increasing concentrations of phytate suggested that the absorption of zinc is inversely correlated to the phytate content of the diet. There was no threshold level which is required to be surpassed to observe an effect, as has been suggested for iron (Lonnerdal *et al.*, 1988). Therefore, any reduction in dietary phytate content is likely to result in an improvement in zinc absorption.

Studies have shown that the hexaphosphate and pentaphosphate forms inhibited zinc absorption in a rat pup model, whereas the tetraphosphate and triphosphate forms had no significant effect (Lönnerdal et al. 1989). Subsequent studies in human subjects confirmed these findings (Sandström and Sandberg 1992).

It has been known that phytic acid has an antineoplastic activity. Since phytic acid can inhibit zinc absorption and result in zinc deficiency, and zinc deficiency leads to increased apoptosis, phytic acid may have an antitumour activity by inducing apoptosis of tumour cells via a decrease in intracellular zinc.

1.8.2 Phytic acid and colorectal cancer

As a component of dietary fibre, phytic acid has been studied extensively for its chemopreventive properties against colon carcinogenesis in laboratory animal models. Phytic acid reduced the number of colon tumours induced by the carcinogen azoxymethane as well as the size of tumours (Shamsuddin *et al.*, 1988). It was shown that this inhibition of colon cancer was dose-dependent (Ullah and Shamsuddin, 1990). Furthermore, dietary phytic acid reduced the incidence of colonic aberrant crypt foci in rats (Challa *et al.*, 1997; Jenab and Thompson, 1998; Pretlow *et al.*, 1992). Oral administration of phytic acid was shown to inhibit colon carcinogenesis in rodents not only in the initiation but in post-initiation stages as well (Jenab and Thompson, 1998). Moreover, the study by Jenab and Thompson (2000) suggested that part of the protective action of wheat bran is due to phytic acid and that exogenous phytic acid when added to low fibre diet can increase both apoptosis and differentiation in colonocytes in vivo.

Also, studies showed that phytic acid was protective against tumours from breast (Hirose *et al.*, 1994; Shivapurkar *et al.*, 1996; Vucenik *et al.*, 1993; Vucenik *et al.*, 1995), liver (Hirose *et al.*, 1991), lungs (Estensen and Wattenberg, 1993), prostate (Shamsuddin and Yang, 1995) and other organs (Shamsuddin, 1995).

Although phytic acid can normalise the increased mitotic rate induced by carcinogen, it does not affect the mitotic rate in normal animals (Shamsuddin *et al.*, 1988;

Shamsuddin and Ullah, 1989; Shamsuddin *et al.*, 1989; Ullah and Shamsuddin, 1990). Another property of phytic acid is that it causes an enhanced differentiation as well as an inhibition of cell proliferation. Reversion of the malignant phenotypes to more mature phenotypes has been seen at least in the human erythroleukemia cell line K-562 (Shamsuddin *et al.*, 1992) and human colorectal cancer cell line HT-29 (Yang and Shamsuddin, 1995). The response of different cell lines to phytic acid varies. The cells of hematopoietic linage (eg. K-562, HL-60 cells) are highly sensitive to phytic acid while those of epithelial and mesenchymal lines require higher concentrations (Arnold *et al.*, 1995; Babich *et al.*, 1993; Shamsuddin *et al.*, 1992; Shamsuddin and Yang, 1995).

It is known that phytic acid acts as an antioxidant to reduce the rate of cell proliferation and to augment the immune response, however, the mechanism of the anti-tumour effect remains unclear.

1.8.3 Mechanism of anti-tumour activity of phytic acid

Studies have shown that phytic acid is rapidly absorbed from stomach and upper small intestine by rats. Ion exchange chromatography demonstrated the presence of inositol and $InsP_{1-6}$ in gastric epithelial cells as early as 1 h after intragastric ^{3}H-$InsP_6$ administration. The presence of $InsP_6$ within the gastric epithelial cells suggests that the intact molecule was perhaps transported into the cell where it was rapidly dephosphorylated (Sakamoto *et al.*, 1993). The metabolised $InsP_6$, in the form of inositol and $InsP_1$ is transported via plasma and reaches distant organs as well as tumours. In rats, the urinary metabolites of $InsP_6$ are inositol and $InsP_1$ (Sakamoto *et al.*, 1993). However, in humans, the small intestine has been shown to have very little ability to dephosphorylate $InsP_6$ (Iqbal *et al.*, 1994), and thus $InsP_6$ should be even more effective

against cancers in humans than in rats. 1-3% of total administered $InsP_6$, in humans, is excreted in the urine as $InsP_6$.

Uptake studies also showed that phytic acid instantaneously began to accumulate in the cytosol of cancer cells after incubation in vitro, the rate of accumulation varying for the different types of cells (Vucenik and Shamsuddin, 1994). The rate and pattern at which the cells metabolised $InsP_6$ also varied depending on the cell types; YAC-1 and K-562 cells contained only the lower InsPs, whereas HT-29 human colorectal carcinoma cells had inositol and $InsP_{1-6}$ (Vucenik and Shamsuddin, 1994).

Although the mechanism of antitumour action of phytic acid is not clear, evidence available showed that the central pathway of $InsP_6$ action is via control of cell division. Reduction of tumour cell proliferation has been shown both in vivo and in vitro. ^{3}H-thymidine incorporation studies demonstrated a reduction in DNA synthesis as well. Several possible mechanisms of anti-tumour action of phytic acid have been proposed. Firstly, phytic acid may act by chelating cations, such as Zn^{2+}, since the metalloproteins are important in gene regulation. Secondly, phytic acid competes for binding sites present on the insulin-like growth factor II receptor (Kar *et al.*, 1994). It appears that insulin-like growth factor II receptor is important in the initiation of cellular hyperplasia (MacDonald *et al.*, 1993). By competing with insulin-like growth factor II for receptor binding, phytic acid may antagonise the regulation of insulin-like growth factor II on cellular proliferation. Thirdly, InsP6 profoundly inhibits EGF- or TPA-induced cell transformation and the signal transduction cascade to Erks and AP-1 activation by blocking TPA- or EGF-induced PI-3 K activity, suggesting that the anticarcinogenesis action of $InsP_6$ may be through inhibition of PI-3 K and inhibition of the AP-1 pathway (Huang *et al.*, 1997).

Finally, studies have demonstrated up-regulation of wild type p53 (Saied and Shamsuddin, 1998) and down-regulation of the mutant form (Vucenik *et al.*, 1998). Also, $InsP_6$ up-regulates the expression of p21 in a dose-dependent manner (Saied and Shamsuddin, 1998).

1.9 AIMS OF THESIS

In this thesis, the following issues were adressed, with a view to better understanding the mechanism of effect of butyrate on apoptosis and how it might interact with other exogenous factors such as drugs (NSAIDs) and other dietary components.

1. The relationship of butyrate-induced growth arrest to apoptosis in colorectal cancer cells.

2. The relationship of p21 and pRb to butyrate-induced growth arrest and apoptosis of colorectal cancer cells.

3. The interaction of butyrate and NSAIDs on apoptosis of colorectal cancer cells.

4. The interaction of butyrate and dietary zinc on apoptosis of colorectal cancer cells, mainly focusing on the effect of depletion of intracellular zinc levels on caspase activation and regulation of p21 in colorectal cancer cells.

5. The interaction of butyrate and phytic acid on apoptosis of colorectal cancer cells.

CHAPTER 2

MATERIALS AND METHODS

2.1 MAJOR MATERIALS AND THEIR SUPPLIERS

2.1.1 Chemicals and reagents

The following chemicals and reagents were purchased from Sigma Chemical Co. Ltd, St Louis, MO.

Albumin, Arachidonic acid, Ampicillin,

Dithiothreitol, Hepes, herring sperm DNA

EDTA, EGTA, Dimethyl sulfoxide (DMSO)

Glycine, Hoechst dye 33258 and Hoechst dye 33342

Kanamycin, Nordihydroguaiaretic acid (NDGA),

Nonidet P-40 (NP-40), sucrose, Phytic acid, TPEN, Trizma base

The following chemicals and reagents were purchased from BDH, Poole, England.

Formaldehyde

Methanol

Sodium butyrate

Sodium dodecyl sulphate (SDS)

Zinc sulphate

The following chemicals and reagents were purchased from Kamiya Biomedical Co., Tukwila, WA.

zDEVD-AFC (z-asp-glu-val-asp-7-amino-4-trifluoro-methyl-coumarin),

zVAD-fmk (z-val-ala-asp-fluoro-methyl-ketone);

zDEVD-fmk (z-asp-glu-val-asp-fluoro-methyl-ketone);

VEID-AFC (val-glu-ile-asp-7-amino-4-trifluoro-methyl-coumarin)

The following chemicals and reagents were purchased from LKB-Produkter AB, Bromma Sweden.

N,N,N',N'-tetramethyl-ethenediamine (TEMED)

β-mercaptoethanol

The following chemicals and reagents were purchased from Bio-Rad laboratories, Hercules, CA.

Ammonium persulfate

Acrylamide/bisacrylaminde (29:1)

The following chemicals and reagents were purchased from ICN, Aurora, OH.

CHAPS, EDTA/trypsin, glutamine

Penicillin/streptomycin

The following chemicals and reagents were purchased from Amersham Life Sciences, Sydney, Aust.

ECL Western Blotting reagent,

Rainbow high mol wt markers

The following chemicals and reagents were purchased from other companies.

Agarose DNA grade (Progen industries, Queensland, Aust)

Complete protease inhibitor cocktail (Boehringer Mannheim, Germany)

Diphenylamine (Ajax, Auburn, Victoria, Aust)

Ethidium Bromide (Boehringer Meinheim)

Gentamicin (Pharmacial & Upjohn Pty Limited, Bently, Aust)

Low melt agarose (Progen industries, Queensland, Aust)

dNTP's (Pharmacia Biotech, Piscataway, NJ)

Sulindac sulfide and sulfone (Cell Pathways, Inc, Horsham, PA)

TRIZOL Reagent (Gibco BRL, Life Technologies Pty Ltd, Mulgrave, Vic, Aust)

Tween 20 (Ajax, Auburn, Victoria, Aust)

Zinquin was a gift of Dr A. Ward, Department of Chemistry, University of Adelaide

All other reagents were reagent-grade, unless indicated.

2.1.2 Radiochemicals

(α-^{32}P) dCTP, 3000Ci/mM (Geneworks, Adelaide, SA, Aust)

2.1.3 Kits

DC protein assay kit (Bio Rad Lab, Hercules, CA)

Gigaprime DNA Labelling Kit (Geneworks, Adelaide, SA, Aust)

CONCERT^{TM} High Purity Plasmid Midiprep System (Gibco BRL, Life Technologies Pty Ltd, Mulgrave, Vic, Aust)

Wizard PCR preps DNA purification system (Promega Corporation, Sydney, Aust)

2.1.4 Enzymes

EcoR I (NewEngland Biolabs, Beverly, MA)

BamH I (NewEngland Biolabs, Beverly, MA)

T4DNA ligase (NewEngland Biolabs, Beverly, MA)

Ampli Taq-gold (PE applied Biosystem, Foothill, CA)

2.1.5 Buffers and solutions

5xelectrode (Running) buffer: Tris base 1.5%, glycine 7.2% and SDS 0.5%

10xHanks Balanced Salt Saline (HBSS) buffer (ICN Biomedicals, Aurora, Ohio)

NP-40 lysis buffer: 5 mM Tris-HCl pH 7.5, containing 5 mM EDTA and 0.5% NP-40

Protease buffer: 50mM Hepes, 10% sucrose, 10 mM DTT, 0.1% CHAPS, pH 7.4

4x SDS-loading buffer: 250 mM Tris-HCl pH 6.8, 40% v/v glycerol, 8% (w/v) SDS, 20% v/v 2-mercaptoethanol and 0.5% w/v bromophenol blue

TNE buffer: 10mM Tris-HCl, pH 7.4, containing 100mM NaCl and 1mM EDTA

Phosphate Buffered Saline (PBS) buffer: 80mM Na_2HPO4, 20mM NaH_2PO_4 and 100mM NaCI, PH7.5.

Tris Buffered Saline (TBS) buffer: 20 mM tris base, 137 mM NaCI, adjust pH to 7.6 with 1M HCI.

Transfer buffer: 25mM Tris, 192mM glycine, 20% v/v methanol, pH 8.3

Separating gel formula (12%): Distilled water 3.35ml, 1.5M Tris-HCI (pH8.8) 2.5ml, 10% (w/v) SDS stock 100µl, acrylamide/Bis (30% stock) 4ml, 10% ammonium persulfate (fresh daily) 50µl, TEMED 5µl

Stacking gel formula (4%): Distilled water 6.1ml, 0.5M Tris-HCI (pH8.8) 2.5ml, 10% (w/v) SDS stock 100µl, acrylamide/Bis (30% stock) 1.3ml (degassed for 15 min at room temperature), 10% ammonium persulfate (fresh daily) 50µl, TEMED 10µl.

2.1.6 Bacterial growth media

All liquid media were prepared using Milli-Q water and sterilised by autoclaving. The formulas of the various media were as follows:

L-broth: 1% Bacto tryptone, 0.5% Bacto yeast extract, 1% NaCI, pH7.4

L-Agar: L-broth, 1.5% Bacto agar

L-Amp: L-broth, 1.5% Bacto agar, ampicillin (50 μg/ml)

2.1.7 Bacterial strains

JM109 competent cells, subcloning efficiency (Promega Corporation, Sydney, Aust). Genotype: *end*A1, *rec*A1, *gyr*A96, *thi*, *hsd*R17 (r_k-, m_k^+), *rel*A1, *sup*E44, δ (*lac-pro*AB), (F', *tra*D36, *pro*AB, *lac*I^qZ δ M15).

2.1.8 Plasmid vectors

PEGFP-N1 (Clontech, Palo Alto, CA).

2.1.9 Synthetic oligonucleotides

The following primers were designed and synthesized by GibcoBRL, Melbourne, Australia.

5p21E gcgaattctgatgtcagaaccggctggggatg

3p21B2 agacggatcccggtccacatggtcttcctc

3p21B gtggggatcccggggcttcctcttggagaag

2.1.10 Tissue culture solutions

RPMI-1640 medium (Sigma Chemical Co. Ltd, St Louis, MO)

Foetal bovine serum (Biosciences, Sydney, Aust)

Trypsin-EDTA (Sigma Chemical Co. Ltd, St Louis, MO)

2.1.11 Cell lines

Colorectal cancer cell lines: LIM1215 was a gift from Dr R. Whitehead (Ludwig Institute Melbourne, Australia). SW480 was a gift from Dr A. Dobrovic, Department of Haematology, The Queen Elizabeth Hospital, Woodville, South Australia.

2.1.12 Antibodies

Monoclonal mouse antibody against human p21, Clone 6B6, 1/333 dilution (Pharmingen, Franklin Lakes, NJ).

Monoclonal mouse antibody against human Rb (Santa Cruz, CA).

Polyclonal rabbit antibody against human p27 (Calbiochem-Novabiochem Pty, NSW, Aust).

Sheep anti-mouse IgG peroxidase conjugate, Fab fragment (Boehringer Mannheim, Germany).

Donkey anti-rabbit Ig peroxidase conjugate, whole antibody (Boehringer Mannheim, Germany).

2.1.13 Miscellaneous

Fluorimeter-grade disposable cuvettes (Greiner, Kremsmunster, Austria)

25 cm^2 or 75 cm^2 vented tissue culture flasks, or 6-well plates (Sarstedt, Newton, NC)

Hybond N^+ nylon membranes (Amersham, Castle Hill, NSW, Aust)

Immobilon-P PVDF membrane (Millipore, Sydney, Aust)

2.2 GENERAL METHODS

2.2.1 Cell cultures

Cells were cultured in RPMI 1640, HEPES-buffered, pH 7.4, supplemented with glutamine (2 mM), penicillin (100 IU/ml), streptomycin (100 μg/ml), gentamicin (160 μg/ml) and 10% foetal bovine serum (FBS) in a humidified atmosphere containing 5% CO_2. Experiments were performed either in 25 cm^2 or 75 cm^2 vented tissue culture flasks, or 6-well plates. Cells were allowed to attach and grow for 2-3 days prior to exposure to

test reagents. For cultures with butyrate, stock solutions (400 mM) in RPMI were made fresh each day and diluted into the cell suspension to give the desired final concentration (in most experiments this was 1 or 4 mM).

2.2.2 Determination of morphological changes of apoptosis

For morphological assessment, cells were examined by bright field microscopy using a 100x oil immersion lens (Olympus, Tokyo, Japan). Apoptotic cells were distinguishable from normal cells by their nuclear fragmentation, presence of apoptotic bodies, decreased size and, sometimes, intense membrane blebbing. Cells with one or more of these properties were scored as positive. In most cases, they excluded trypan blue. In some experiments, hoechst dye 33342 was added to a final concentration of 100 μg/ml and cells analyzed by fluorescence microscopy for chromatin segregation, using an Olympus BH2 microscope, equipped with epifluorescence, UV B dichroic mirror for low wavelength excitation, and connected to a Panasonic CCTV video colour camera suitable for low light fluorescence with integration capabilities, and a computer work station. Both fluorescence images and corresponding bright field microscope images were captured and stored.

2.2.3 Measurement of DEVD-caspase activity

DEVD-caspase was assayed by cleavage of zDEVD-AFC (z-asp-glu-val-asp-7-amino-4-trifluoro-methyl-coumarin), a fluorogenic substrate based on the peptide sequence at the caspase-3 cleavage site of poly (ADP-ribose) polymerase (Nicholson *et al.*, 1995). Cells (1-2x10^6 per well or 5x10^6-10^7 per flask) were cultured with test reagents, washed once with 5 ml of HBSS and resuspended in 1ml of NP-40 lysis buffer (as for

DNA fragmentation). After 15 min in lysis buffer at 4°C, insoluble material was pelleted at 15,000g and an aliquot of the lysate was tested for protease activity. To each assay tube containing 8 μM of substrate in 1 ml of protease buffer was added 20 μl of cell lysate. After 4.5 h at room temperature, fluorescence was quantified (Exc 400, Emis 505) in a Perkin Elmer LS50 spectrofluorimeter. Optimal amounts of added lysate and duration of assay were taken from linear portions of curves as determined in preliminary experiments. One unit of caspase activity was taken as one fluorescence unit (at slit widths of 5 nm) per 4.5 h per mg protein.

2.2.4 Measurement VEID-caspase (caspase-6) activities

VEID-caspase was assayed by cleavage of VEID-AFC (val-glu-ile-asp-7-amino-4-trifluoro-methyl-coumarin), a fluorogenic substrate based on the peptide sequence at the caspase-6 cleavage site of lamin (Takahashi *et al.*, 1996). Cells ($1\text{-}2\times10^6$ per well or $5\times10^6\text{-}10^7$ per flask) were cultured with test reagents, washed once with 5 ml of HBSS and resuspended in 1 ml of NP-40 lysis buffer (as for DNA fragmentation). After 15 min in lysis buffer at 4°C, insoluble material was pelleted at 15,000g and an aliquot of the lysate was tested for protease activity. To each assay tube, 20 μl of cell lysate was added, followed by 1 ml of protease buffer containing 8 μM of substrate. After 4.5 h at room temperature, fluorescence was quantified (Exc 400, Emis 505) in a Perkin Elmer LS50 spectrofluorimeter. Optimal amounts of added lysate and duration of assay were taken from linear portions of curves as determined in preliminary experiments. One unit of caspase activity was taken as one fluorescence unit (at slit widths of 5 nm) per 4.5 h per mg protein.

2.2.5 Apoptotic DNA fragmentation

To assay DNA fragmentation, cells (1-2x10^6 per well or 5x10^6-10^7 per flask) were lysed at 4°C in 1 ml of NP-40 lysis buffer and centrifuged at 13,000g for 10 min at 4°C. Supernatant fractions containing low molecular weight DNA fragments were assayed for DNA by a fluorometric technique (Teare *et al.*, 1997). Hoechst dye 33258 was dissolved in deionized water to a concentration of 1 mg/ml and stored at 4°C. Prior to use, 1 µl of dye was added per 10 ml of TNE buffer. Aliquots of lysate (20-50 µl) were placed into fluorimeter-grade disposable cuvettes (Greiner) and 1 ml of diluted dye solution added. Fluorescence was measured at an excitation wavelength of 356 nm and emission wavelength of 458 nm (slit widths 10 nm) in a Perkin-Elmer fluorescence LS50 spectrophotometer. Herring sperm DNA was used to derive a standard curve.

2.2.6 Quantification of Zinquin fluorescence by image analysis

Cells were grown to semi-confluence on sterile glass coverslips in 6-well plates for 48 h, prior to the addition of Zinquin. Coverslips were then washed in PBS, and incubated with 25 µM Zinquin in PBS, for 30 min at 37°C. Coverslips were inverted on microscope slides. Fluorescence was examined using the same image analysis system as described in Section 2.2.2. Both fluorescence images and corresponding phase contrast microscope images were captured and stored using a 20x objective lens. Using the VideoPro image analysis system, lines were drawn around the borders of cells and fluorescence intensity within the borders was measured. For all images, background illumination in an area not occupied by cells was subtracted from the readings.

2.2.7 Cell cycle analysis

Cells were removed from culture dishes by trypsinisation, collected by centrifugation, resuspended in ice-cold PBS and then fixed in absolute methanol for at least 30 minutes. Cells were then washed in PBS containing 0.5% Tween 20, followed by two washes in PBS containing 2% FBS. After washing, cells were resuspended in PBS/2% FBS containing 40 μg/ml RNase A and incubated for 20 min at 37°C. Finally, cells were washed in PBS/2%FBS and resuspended in PBS containing propidium iodide (20 μg/ml). The stained nuclei were analysed using a flow cytometer (Epics Profile, Coulter). Cell cycle distribution was based on 2N and 4N DNA content. Cells with less than 2N DNA content were indicative of apoptotic cells.

2.2.8 RNA extraction and Northern blot analysis

For Northern blot analysis, cells were seeded at a density of 3-5 x 10^5 cells/well, allowed to attach for 2 days and incubated in the presence or absence of 4 mM butyrate. Total RNA was isolated at the indicated times using the TRIZOL Reagent according to the manufacturer's instructions. Total RNA (10 μg per lane) was electrophoresed in formaldehyde/1% agarose gels, transferred to Hybond N^+ nylon membranes, and immobilised by UV cross-linking. Membranes were pre-hybridised for 3 hrs at 42°C in 1 M NaCl, 1% SDS, 10% dextran sulphate, 50% formamide, 100 μg/ml of heat-denatured herring sperm DNA. They were then hybridised with $p21^{Waf1/Cip1}$cDNA (a kind gift from Dr Helena Richardson, The University of Adelaide, SA, Australia), which was radiolabeled with (α-^{32}P) dCTP by random priming using the Giga prime kit. To allow quantification of mRNA signals, the same filters were reprobed with a 450 bp ^{32}P-labeled PCR-generated DNA fragment of human glyceraldehyde 3-phosphate dehydrogenase

(GAPDH). Following hybridisation for 16 h at 42°C, filters were washed at high stringency and resulting signals analysed using the PhosphorImager SF (Molecular Dynamics Inc.).

2.2.9 Western Blotting

Aliquots of cells were lysed in 0.5% NP-40 lysis buffer containing complete protease inhibitor cocktail and stored at -20°C until assayed. Protein concentration was measured using the DC protein assay kit. One volume of 4x SDS-loading buffer was added to three volumes of lysate and mixtures were denatured at 100°C for 5 min. Equal protein amounts (18-36 μg) were loaded into wells and electrophoresed on 7.5% (for Rb) or 12% (for p21, p27, cpp32) minigels at 200 volts for 45 min. Proteins were blotted onto Immobilon-P PVDF membrane. Membranes were preblocked with 3% powdered milk in tris-buffered saline (TBS)/0.1%Tween-20, 3% bovine serum albumin for 60min at room temperature. Primary antibodies used were mouse monoclonal antibodies against human p21 (Clone 6B6, 1/333 dilution, Pharmingen) and human Rb (1/333) and polyclonal rabbit antibodies against human p27 (1/500 dilution). Secondary antibody was either sheep anti-mouse IgG peroxidase conjugate (1/1000 dilution, Fab fragment) or donkey anti-rabbit Ig peroxidase conjugate (1/1000 dilution, whole antibody). Primary antibodies were added for 60 min and secondary antibodies for 45 min at room temperature. Membranes were then soaked in ECL Western Blotting reagent and bands detected by chemiluminescence. Rainbow high mol wt markers were used for determination of mol wt.

2.2.10 Polymerase chain reaction (PCR)

Target DNA, PCRII-p21 (50 ng) was placed in a 0.5ml reaction tube in a total volume of 20 µl containing 50 ng of each of the oligonucleotide primers, 0.2 mM of dNTP's, 0.9 mM of $MgCl_2$, 1 unit of polymerase and 2 µl of Taq polymerase buffer (10x). The DNA was then denatured by heating the reaction to 94 °C for 5 min, followed by annealing at the designated temperature, dependent on the specific pair of primers being used, and elongation at 72 °C for the appropriate time, dependent on the length of PCR product expected. In general, 35 cycles were performed and annealing temperatures was 55 °C. All PCR reactions were performed in a thermal cycler (PC-960C Cooled Thermal Cycler, Corbett Research). PCR products were analyesd by electrophoresis on argarose gels containing ethidium bromide.

2.2.11 Restriction enzyme digestions

0.5-1µg of plasmid DNA or PCR products were incubated with 5-10 units of each of appropriate restriction enzymes (*Eco*RI and *Bam*HI) for a minimum of 1 h in the buffer conditions specified by the manufacture. Restriction fragments were electrophoresed on 1% mini-agarose gels in TAE buffer. In preparative digests, 5-10 µg of DNA was used in a total volume of 30-100µl and the desired DNA fragments were isolated as described below.

2.2.12 Preparation of DNA restriction fragments

The plasmid DNA or PCR products were digested with the appropriate restriction enzymes as described above (2.2.11). Fragments were isolated from horizontal 0.8-1.5% low melting point agarose gels depending on the size of the DNA restriction fragment.

Bands representing restriction fragments were visualised under UV light following staining with ethidium bromide, and the appropriate fragments excised from the gel using a sterile scalpel blade. Gel slice was placed at 72°C until melted. Restriction fragments was purified by the Wizard (PCR preps) DNA purification kit (Promega) following the instructions supplied by manufacturer.

2.2.13 Ligation of DNA inserts into plasmid vectors

The DNA insert and the appropriate plasmid vector (100ng) were combined in the molar ratios of 1:1, 1:3 and 3:1, in a 20µl reaction volume with 15 units of T4 DNA ligase and in the buffer supplied by the manufacturer. The reactions were routinely incubated overnight at 4°C. A control ligation of vector without insert was usually set up and included in the subsequent transformations to determine background levels of uncut or recircularised vector. The PEGFP-N1 vector was used for the direct cloning of PCR products according to the supplied protocol.

2.2.14 Transformation of E. coli

Plasmid vectors (25ng) or DNA ligation reactions corresponding to half the volume of the ligation mixture were mixed with 100µl of competent bacteria JM109 cells and incubated on ice for 20min. The cells were then heat-shocked at 42°C for 2-5 min, placed in 900 µl of L-broth and incubated at 37 °C with shaking (225 rpm) for 1.5 h. 100 µl of transformation reaction was plated using a sterile spreader onto L-agar containing 50 µg/ml of kanamycin. The agar plates were routinely incubated overnight at 37°C.

2.2.15 Plasmid DNA preparation

For plasmid preparation, recombinant clones, were grown overnight in 50ml of L-broth supplemented with 50ug/ml of kanamycin. The cells were pelleted by centrifugation at 4000 rpm for 30min at 4°C and plasmids isolated using Gibco BRL CONCERT™ High Purity Plasmid Midiprep System according to the instructions supplied by manufacturer.

2.2.16 Transfection of p21 and p15 in LIM1215 cells and SW480 cells

DNA transfections were performed with either vector DNA or recombinant plasmids using LIPOFECTAMINE™ 2000 Reagent following the manufacturer's instruction. Briefly, 8-10x10^5cells were seeded into 6-well plates; they were 90-95% confluent on the day of transfection. For each well, 4-5 μg DNA plasmids were diluted into 250 μl DMEM medium without serum. 5-15 μl of LF2000 Reagent was diluted into 250 μl DMEM and incubated for 5min at room temperature. The LF2000 Reagent was combined with the DNA and incubated at room temperature for 20min to allow DNA-LF2000 Reagent complexes to form. The mixture was added directly to each well, mixing gently by rocking the plate back and forth. The cells were incubated at 37°C in a CO_2 incubator for a total of 72 h. These cells were used in apoptosis experiments.

2.2.17 Protein assay

Protein concentration was measured by the DC protein assay kit (Bio Rad Laborateries, Hercules, CA) in accordance with the manufacturer's instructions.

2.2.18 Experimental design

All experiments were repeated a minimum of three times. Typical experiments are described or data were pooled, as indicated in Text. Statistical significance was determined by the student *t*-test and is indicated in text or legends to Figures.

CHAPTER 3

THE EFFECT OF BUTYRATE ON CELL CYCLE PROGRESSION AND APOPTOSIS OF COLORECTAL CANCER CELLS

3.1 INTRODUCTION

There are a range of different strategies available for prevention of CRC which involve intervening in the multistage process leading to invasive tumour cells (Young, 1996). Colorectal tumorigenesis is strongly influenced by environmental factors, especially products of carbohydrate fermentation in the colonic lumen (Cho and Vogelstein, 1992; Young, 1994; Young, 1991). CRCs may be exposed to these substances from the time the initial clone of cancer cells emerges. Of particular importance in protection are dietary polysaccharides (eg wheat bran) which exert their inhibitory effects at the aberrant crypt stage of tumorigenesis (Young *et al.*, 1996). The short chain fatty acid butyrate, a colonic fermentation product of dietary polysaccharides, partly mediates their protective action (McIntyre *et al.*, 1993) and is thought to protect via modulation of expression of colonic epithelial genes involved in cell-cycle arrest, differentiation and apoptotic cell death.

In the cell cycle, temporal order is imposed by the sequential activation of a series of serine/threonine protein kinases. Each functional kinase comprises a regulatory subunit, called a cyclin, and a catalytic subunit, called a cyclin dependent kinase (CDK). There is a growing consensus that the retinoblastoma gene product (Rb) must play some part in the process. In G_0 and early G_1, Rb is hypophosphorylated and migrates as a single band on SDS-PAGE. As the cell progresses through G_1, Rb becomes phosphorylated at multiple sites, culminating in a hyperphosphorylated state in late G_1 that is maintained throughout the remainder of the cycle (Peeper *et al.*, 1994; Weinberg, 1995).

When treated with millimolar concentrations of butyrate, many cell types undergo growth arrest in both the G_1 phase of cell cycle (Wintersberger *et al.*, 1983;

Darzynkiewicz *et al.*, 1980; D'Anna *et al.*, 1980; Barnard and Warwick, 1993) and at G_2/M transition (Gupta *et al.*, 1994; Heerdt *et al.*, 1997; Yamada *et al.*, 1992). However, the molecular mechanisms of these G_1 and G_2 arrests have not been elucidated. Several mechanisms have been proposed. Firstly, histone phosphorylation is closely associated with progression through the cell cycle. Dephosphorylated H1 was correlated with G_1 arrest in butyrate treated Chinese hamster cells (D'Anna *et al.*, 1980). Secondly, increased histone acetylation induced by butyrate was associated with the G_1/S arrest (Kruh, 1982). Finally, butyrate can also cause dephosphorylation of the Rb protein in mouse fibroblasts (Buquet Fagot *et al.*, 1996). Some experiments have shown that the hypophosphorylated Rb induced by butyrate is associated with arrest in G_1 (Vaziri *et al.*, 1998; Yen and Sturgill, 1998) and G_2 (Yen and Sturgill, 1998).

More recently, mM concentrations of butyrate have been shown to kill several cancer cell lines by inducing them to undergo apoptosis (Calabresse *et al.*, 1993; Filippovich *et al.*, 1994; Hague *et al.*, 1993). The relationship between growth arrest, differentiation and apoptosis induced by butyrate is unclear. The mechanisms by which butyrate induces apoptosis are currently being investigated and are the subject of this thesis. One or more cell death genes may be turned on by butyrate since butyrate-induced apoptosis is blocked by inhibitors of RNA and protein synthesis (Medina *et al.*, 1997). Butyrate-induced apoptosis appears to involve activation of caspase-3 (and/or related caspase), since DEVD-caspase activity and caspase precursor processing occur in the cytoplasm of cells preceding morphological changes. These effects were blocked by cycloheximide and by membrane-permeable inhibitors of caspases (Medina *et al.*, 1997). In this thesis, caspase activity as determined using fluorogenic substrate DEVD-AFC will

be referred to as DEVD-caspase rather than caspase 3, since the activity may also be due to caspase 7 and possibly caspase 2. However, caspase activity as determined using specific antibodies on Western blot will be referred to as the specific caspase.

The aim of the experiments described in this chapter was to investigate, in butyrate treated CRC cells, the relationships between growth inhibition and 1) cell cycle changes, 2) phosphorylation state of Rb, and 3) apoptosis, as judged by morphological changes, DEVD-caspase activation and DNA fragmentation. Cell lines were used for these experiments because of their homogeneity and ready availability in large numbers. Cell lines used in these experiments included LIM1215 cells, which contain wild-type p53 (Agapova *et al.*, 1996) and SW480 cells which contain mutant p53 and express elevated levels of mutant p53 protein (Rodrigues *et al.*, 1990).

3.2 RESULTS

3.2.1 Induction of growth arrest and apoptosis by butyrate in LIM 1215 and SW480 cells

To determine the effect of butyrate on the growth of colorectal cancer cells, LIM 1215 cells were seeded into six-well plates and treated with physiologically relevant concentrations (0.5-8 mM) of sodium butyrate, 2 days after seeding. Every day, fresh medium and butyrate were supplied and attached cells (viable cells) were harvested by trypsinization and counted. They were then assayed at daily intervals for 5 days (days 2-7 after seeding) for changes in adherent cell number (as a measure of viable cells). Fig 3.1 shows that growth was retarded at all concentrations of butyrate tested, but particularly at concentrations $\geq$ 1 mM. Five days after addition of 4 or 8 mM butyrate, cell numbers were

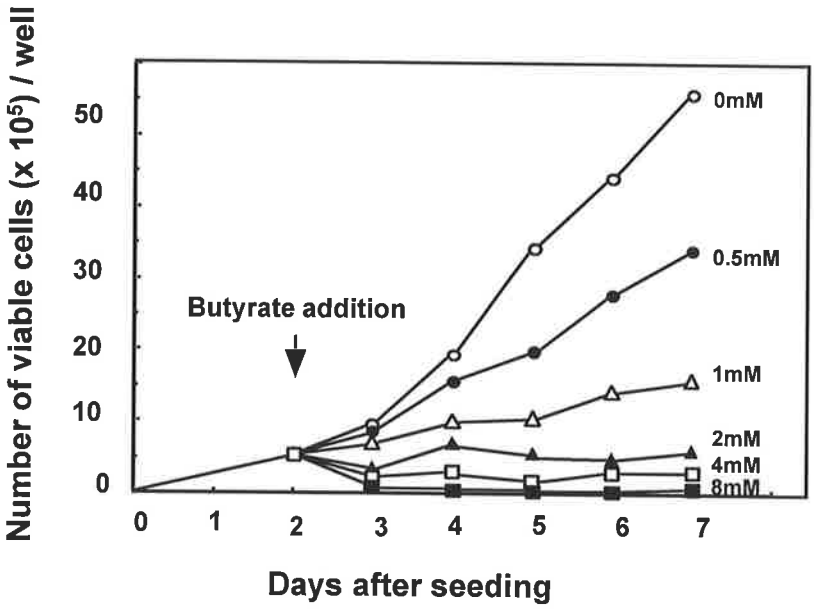


Figure 3.1 The effects of butyrate on growth of LIM1215 cells. LIM1215 cells (4 x 10^5) were seeded into wells at day 0 and varying concentrations of butyrate were added at day 2 (arrow). At daily intervals, thereafter, non-adherent cells were discarded and remaining adherent cells were harvested by trypsinization and counted. Control (open circles); 0.5 mM butyrate (filled circles); 1 mM butyrate (open triangles); 2 mM butyrate (filled triangles); 4 mM butyrate (open squares); 8 mM butyrate (filled squares). Graph shows increasing growth arrest with increasing concentration of butyrate. Typical experiment is shown. Data are means of duplicates.

significantly lower than those at the time of addition of butyrate. Most of this decrease occurred in the first day after addition.

To confirm these effects of butyrate on another colorectal cancer cell line, SW480 cells were treated with 4 mM butyrate for up to 4 days. From Fig 3.2, we can see that total viable cell number in the control increased by approximately 3.5-fold within 4 days; however, following 4 mM butyrate treatment, the total viable cell numbers decreased by about 3-fold after 2 days and 5-fold after 4 days.

To investigate the effects of butyrate on cell cycle progression of LIM1215 cells, flow cytometric analysis with propidium iodide was done to determine cell cycle phase by DNA content. LIM1215 cells were treated with 0-16 mM butyrate for 24 hours (Fig 3.3). At low concentrations of butyrate from 0-3 mM, the proportion of cells in the G_1 phase of the cell cycle gradually increased, accompanied by a decrease of S and G_2/M phase cells, while at high concentrations of butyrate ($\geq$ 4 mM), the number of cells in the G_1 phase of the cell cycle gradually decreased. This was accompanied by an increase of apoptotic cells, as shown by the appearance of an increasing fraction of cells with less than 2N DNA content, preceding the G_1 peak on the fluorescence scans. There was little or no apoptosis with low concentrations of butyrate. These results suggest that growth inhibition of LIM 1215 cells by low concentrations of butyrate is associated with G_1 arrest, whereas higher concentrations of butyrate (such as 4 mM) induce apoptosis.

To further characterize the effect of butyrate on cell cycle progression, flow cytometric analysis was carried out in LIM1215 cells at 3-6 hours intervals. In the first 24 hours of treatment with 1 mM butyrate, there was no alteration in the number of cells in the G_1 phase of the cell cycle, while following 48 hours treatment, a significant increase in

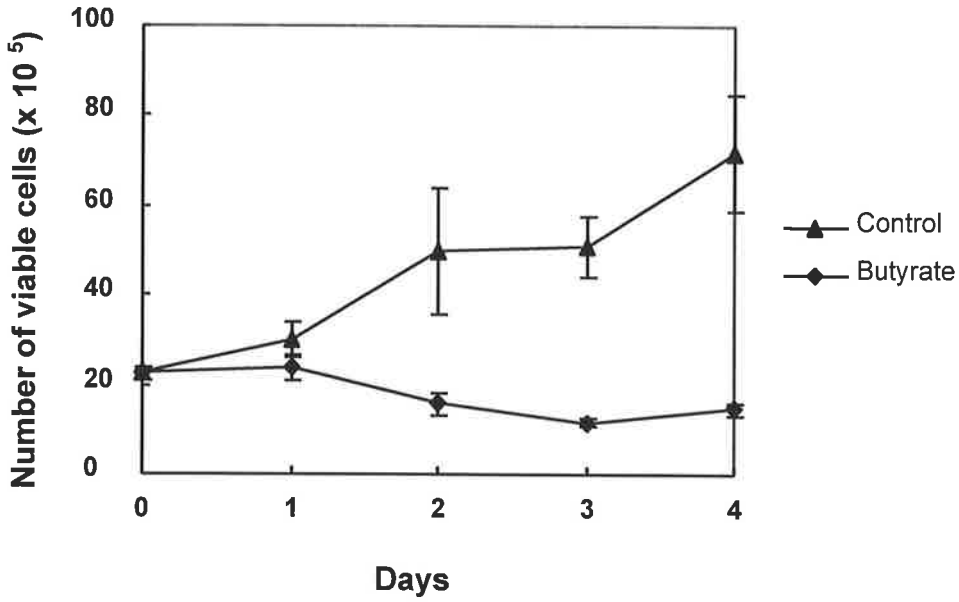


Figure 3.2 The effects of butyrate on growth of SW480 cells. SW480 cells ($5x10^5$) were seeded into wells and, two days after, 4 mM butyrate was added (day 0). At daily intervals thereafter non-adherent cells were discarded and remaining adherent cells were harvested by trypsinization and counted. The graph shows that the growth inhibition of SW480 cells was significant after two days of butyrate treatment.

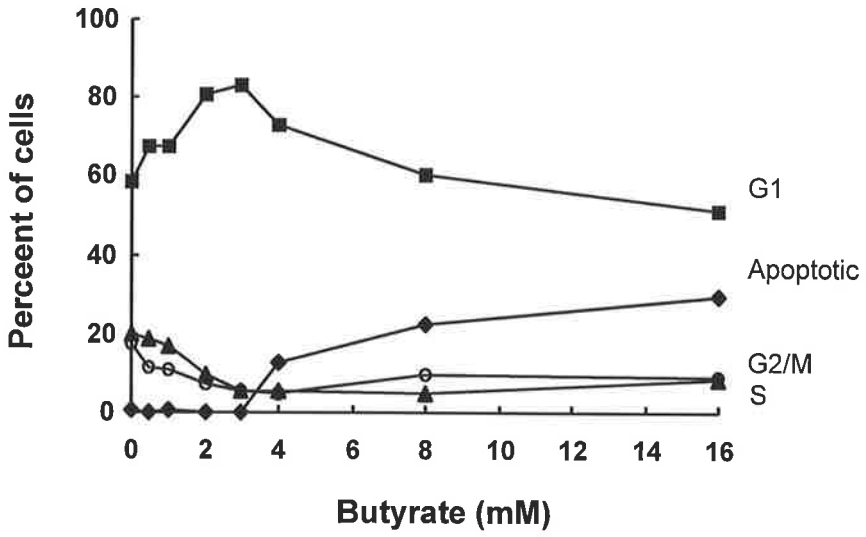


Figure 3.3 Cell cycle analysis of LIM1215 cells following treatment with different concentration of butyrate. LIM1215 cells ($2.4x10^6$) were seeded in 25 cm^2 flasks; two days after, cells were incubated with butyrate from 0-16 mM for 24 hours. Combined non-adherent and adherent cell populations were washed, fixed and analysed by flow cytometry. The graph shows that the number of cells in G_1 phase (filled squares) of the cell cycle, following butyrate treatment from 0.5 mM to 3 mM, increased gradually, accompanied by a decrease of S (filled triangles) and G_2 /M phase (open circles) cells; while at high concentration of butyrate (≥ 4 mM), the number of cells gradually decreased, accompanied by an increase of apoptotic cells (filled rhombuses).

G_1 phase cells occurred, from 59% to 73%. There was no increase in apoptotic cells during this period (Fig 3.4). Treatment with 4 mM butyrate resulted in a progressive depletion of G_1, S and G_2/M phase cells (Fig 3.5), and these were replaced by apoptotic cells beginning at 9-12 hours after addition of butyrate (filled rhombuses in Fig 3.5, left panel), as shown by the increase in fraction of cells with less than 2N DNA content (Fig 3.5, right panel).

3.2.2 Effect of butyrate on phosphorylation states of Rb

Since Rb is an important regulator of the cell cycle, it may mediate the effect of butyrate on cell cycle progression of colorectal cancer cells. To test this, possible alterations in Rb were determined in LIM 1215 cells treated with 4 mM butyrate. In Western blots, Rb runs as a doublet in which the slower migrating band represents hyper-phosphorylated Rb and the faster migrating band represents hypo-phosphorylated Rb (Dou and An, 1998). As seen in Fig 3.6, there was a shift in the phosphorylation state of Rb. Untreated cells showed the characteristic hypo-phosphorylated (pRb) and hyper-phosphorylated (ppRb) forms. Loss of phosphorylated Rb was evident by 6 h and increased thereafter, consistent with p21-mediated inhibition of CDK activity, and cell-cycle arrest.

3.2.3 Induction of apoptosis in butyrate-treated LIM 1215 cells

The decrease in cell numbers at higher concentrations of butyrate suggested that there was not only cell-cycle arrest but also cell death. Flow cytometric analysis revealed an increase in apoptotic cells (filled squares in Fig 3.5, left panel), as shown by the appearance of an increasing fraction of cells with less than 2N DNA content, preceding

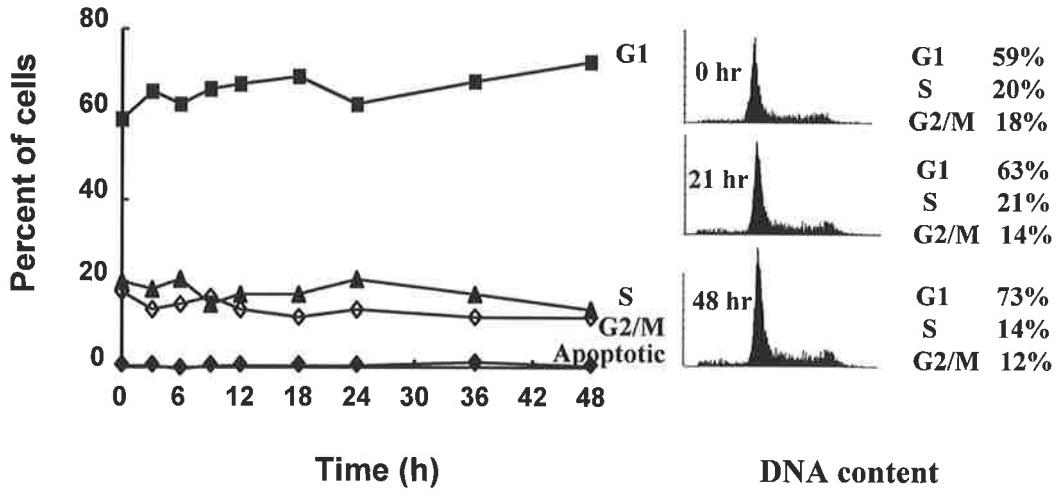


Figure 3.4 Cell cycle analysis of LIM1215 cells following treatment with 1 mM butyrate. Butyrate was added at time 0 and at 3 h intervals combined non-adherent and adherent cell populations were washed, fixed and analyzed by flow cytometery (see Materials and Methods), and the percentage of cells in each phase of the cell cycle determined over time.

Left: G_1 cells (filled squares); S phase cells (filled triangles); G_2/M phase cells (open rhombuses); "sub-G_1" apoptotic cells (filled rhombuses). A significant increase in the number of cells in G_1 phase and no evidence of apoptosis was observed following 48 hour butyrate treatment.

Right: Representative FACS scans at the indicated times.

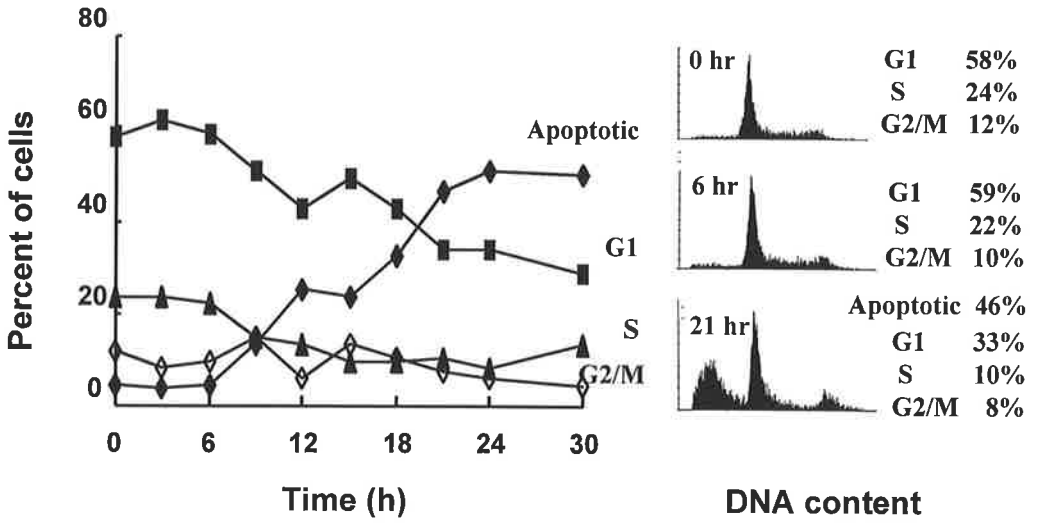


Figure 3.5 Cell cycle analysis of LIM1215 cells following treatment with 4 mM butyrate. Butyrate was added at time 0 and, at 3 h intervals, combined non-adherent and adherent cell populations were washed, fixed and analyzed by flow cytometery (see Methods).

Left: G_1 cells (filled squares); S phase cells (filled triangles); G_2/M phase cells (open rhombuses); "sub-G_1" apoptotic cells (filled rhombuses). Graph shows decreases in cell number at all phases of cell-cycle and replacement by apoptotic cells, beginning after a lag period of ~6 h.

Right: Representative FACS scans at times 0, 6 and 21 h following butyrate treatment. Note the peak of apoptotic cells with less than 2N DNA content at 21 h of treatment.

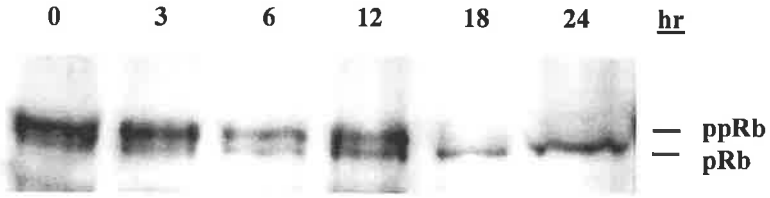


Figure 3.6 Hypo-phosphorylation of Rb in butyrate-treated cells. LIM1215 cells were treated for increasing times with 4 mM butyrate before detection of Rb by western blotting. Equal amounts of protein (18 μg) were loaded. The figure shows loss of slower-migrating hyper-phosphorylated pRb and accumulation of hypo-phosphorylated Rb, particularly between 12 and 18 h. The identification of these two bands as different phosphorylation states of Rb is described in (Dou and An, 1998).

the G_1 peak on the fluorescence scans (Fig 3.5, right panel). Examination of butyrate-treated cells by bright field microscopy showed typical morphological features of apoptosis including the presence of one or more intracellular apoptotic bodies, exclusion of the vital dye trypan blue, membrane blebbing and, reduction in volume (Fig 3.7B), compared with the normal morphology of these cells before treatment with butyrate (Fig 3.7A). Labelling with Hoechst dye 33342 revealed fragmentation of the chromatin into discrete masses in the butyrate-treated cells (Fig 3.7D), compared with the uniform mass of chromatin in untreated cells (Fig 3.7C).

Apoptosis was accompanied by detachment of the cells from the plates. In a typical experiment, incubation of cells for 24 h with 4 mM butyrate resulted in a detachment of 60% of the cells. This floating population consisted of 97% apoptotic cells as detected by morphological features while the adherent fraction contained only 12% apoptotic cells.

3.2.4 Induction of DEVD-caspase activity and DNA fragmentation in LIM 1215 and SW480 cells

Accompanying the above changes were activation of DEVD-caspase and DNA fragmentation, two important determinants of apoptosis.

To determine the effect of different concentrations of butyrate on these two parameters, LIM 1215 cells were seeded in 6 well plates, and after 2 days, they were treated for 24 h with varying concentrations of butyrate from 0 to 16 mM. Cell lysates were prepared and assayed for DEVD-caspase activity. Fig 3.8 shows that DEVD-caspase activity began to increase at 3-4 mM butyrate; after 8 mM butyrate, the curve plateaued. The shape of the curve for DNA fragmentation was similar to that of DEVD-caspase

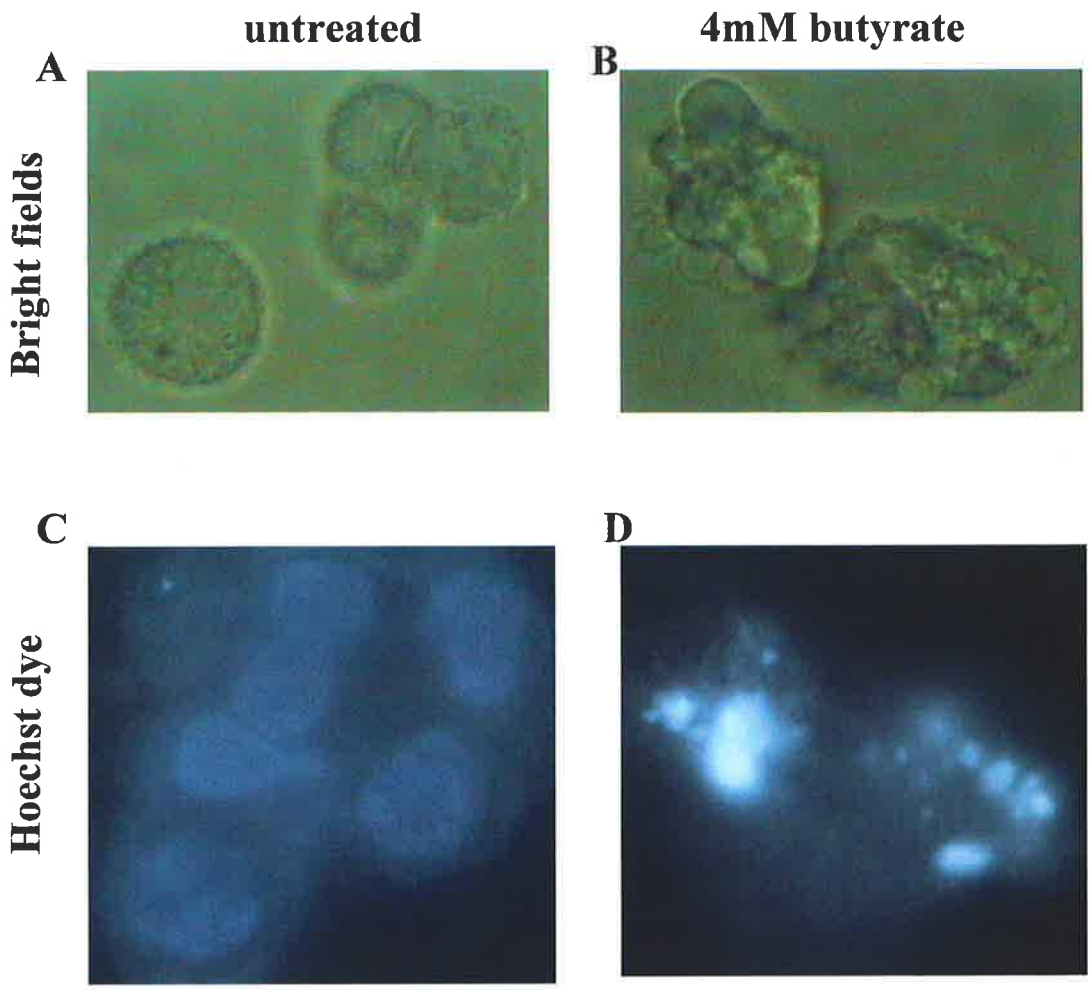


Figure 3.7 Morphological features of apoptosis in LIM 1215 cells treated with 4mM butyrate.

A+B: bright field microscope images of untreated (A) and butyrate treated (B) LIM1215 cells. Initial magnification (2500X).

C+D: UV fluorescence images of Hoechst dye 33342 stained untreated (C) and butyrate treated (D) LIM1215 cells. Initial magnification (2500X).

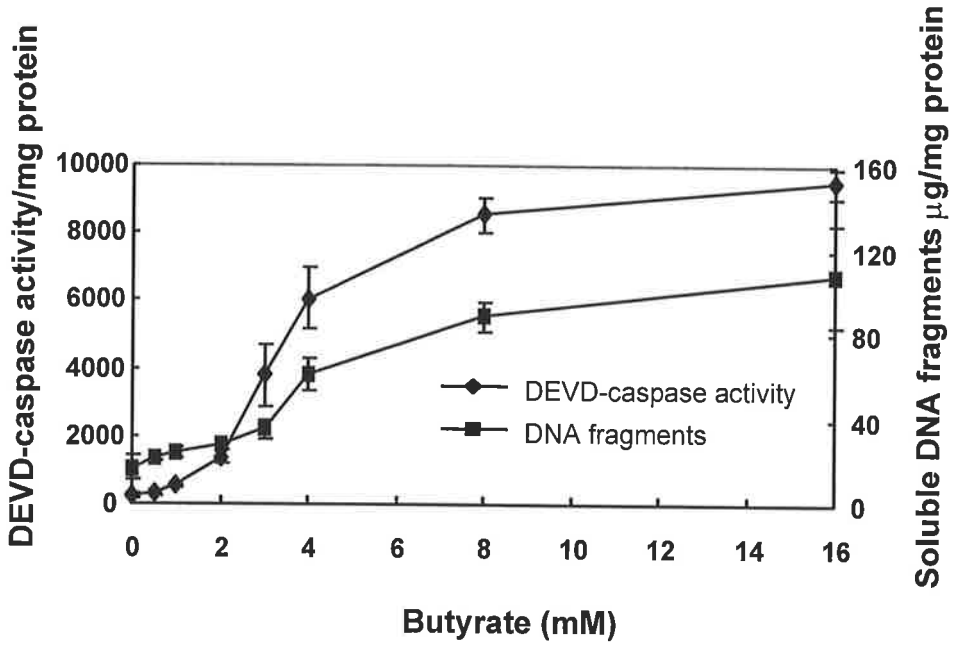


Figure 3.8 Induction of DEVD-caspase activity and DNA fragmentation by butyrate in LIM1215 cells. Cells were treated with varying concentrations of butyrate for 24 h, then harvested and the lysate was assayed for DEVD-caspase activity and DNA fragmentation. This graph shows that induction of DEVD-caspase activity and DNA fragmentation are concentration-dependent; above 8 mM butyrate, the curves plateaued. Data are means of triplicates with error bars indicating standard deviations.

activity. These results are consistent with the results of flow cytometric analysis (Fig 3.5), and confirm that 4 mM butyrate was able to induce apoptosis in LIM 1215 cells.

In SW480 cells, DEVD-caspase activity and DNA fragmentation were also induced by butyrate in a concentration-dependent way (up to 8 mM); above this concentration, the curves plateaued (Fig 3.9). However, considerably less DEVD-caspase activity and DNA fragmentation occurred with SW480 cells than in LIM1215 cells.

To investigate further butyrate-induced apoptosis, the kinetics of DEVD-caspase activity and DNA fragmentation was measured in LIM 1215 cells treated with 4 mM butyrate (Fig 3.10). There was little caspase activity in control cultures, but a marked increase beginning about 9 h after addition of butyrate and peaking at about 15 h. The time-course for DEVD-caspase activation was similar to that for DNA fragmentation (Fig 3.10) and apoptosis (Fig 3.5).

In the results presented so far, DEVD-caspase activity was determined in the combined lysate of the total cell population (adherent cells and cells floating in the medium). To determine whether DEVD-caspase activity was present in both populations, individual lysates were also tested. Although the bulk of DEVD-caspase activity was detected in the floating cell population, significant activity was also present in the adherent cells (data not shown), indicating that activation of DEVD-caspase precedes loss of adhesion in butyrate-treated CRC cells.

Caspase -3 is present as an inactive 32 kDa precursor (cpp32), which is activated by other caspases to yield subunits of 17 and 12 kDa. To confirm further the activation of caspase 3 in butyrate-induced apoptosis, Western blotting was used to detect the cpp32 precursor in LIM 1215 cells treated with varying concentrations of butyrate for 24 hours

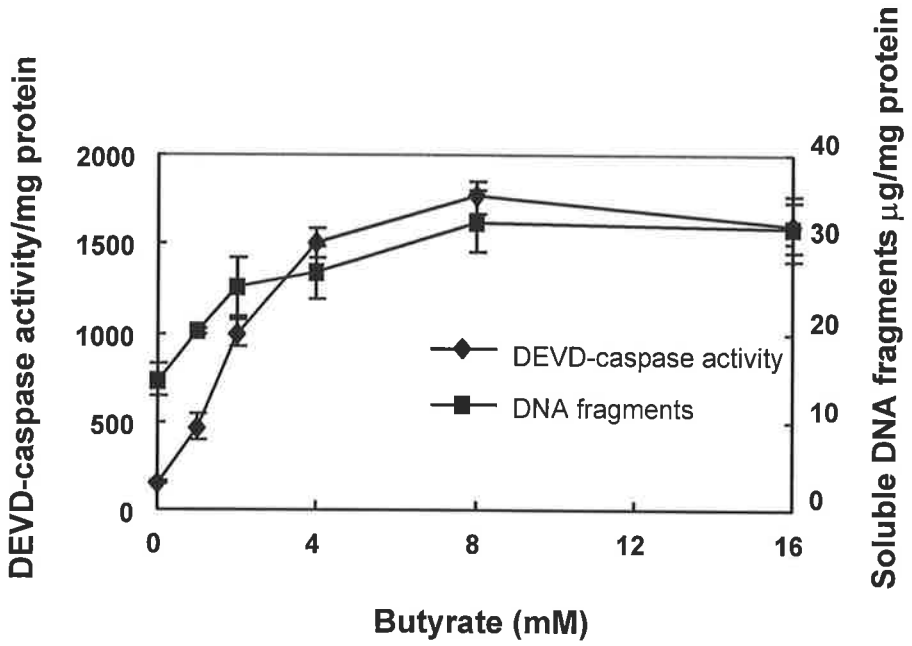


Figure 3.9 Induction of DEVD-caspase activity and DNA fragmentation by butyrate in SW480 cells. Cells were treated with varying concentrations of butyrate for 24 h, then harvested and the lysate was assayed for DEVD-caspase activity and DNA fragmentation. As in Fig 3.8, this graph shows that induction of DEVD-caspase activity and DNA fragmentation have similar butyrate concentration-dependence. However, the extent of both DEVD-caspase activity and DNA fragmentation was only about 20% of that of LIM1215 cells, in the plateau phase of the curve. Data are means of triplicates with error bars indicating standard deviations.

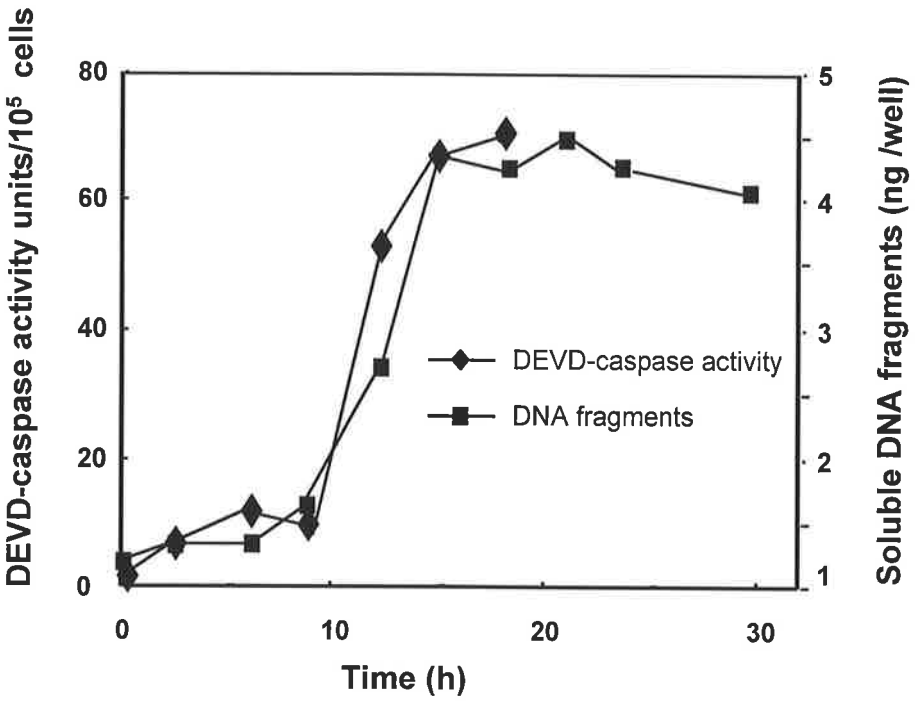


Figure 3.10 Time course of DEVD-caspase activity and DNA fragmentation in butyrate-treated LIM1215 cells. Butyrate (4 mM) was added at time 0 and, at 3 h intervals, combined non-adherent and adherent cell populations were washed, lysed in NP-40 lysis buffer and assayed for DEVD-caspase activity (filled rhombuses) and DNA fragmentation (open squares). Data are means of triplicates.

(Fig 3.11). The antibody against caspase 3 that we used in this study could not detect the 17 kDa or 12 kDa subunits. Intensity of the cpp32 band decreased with higher concentrations of butyrate (16 mM), but no apparent decrease occurred with 4 mM butyrate. This data is not consistent with that of DEVD-caspase activity where 4 mM butyrate induced substantial DEVD-caspase activity (Figure 3.8). A possible reason for this is given in the discussion.

3.3 DISCUSSION

In this chapter, colorectal cancer cell lines LIM1215 and, in some experiments SW480, were used to test the effects of butyrate on cell growth and apoptosis. Butyrate induced both growth arrest and apoptosis, but the relationship between the two is still unclear. Low concentrations of butyrate (< 4 mM) resulted in growth arrest without apoptosis, while higher concentrations (≥ 4 mM) induced apoptosis, without obvious effects on growth arrest. This suggested that butyrate-induced apoptosis was not the direct consequence of the growth arrest, although growth arrest may be a pre-requisite step.

The effects on growth arrest and apoptosis paralleled in time the shift in the phosphorylation state of the cell-cycle regulatory retinoblastoma protein. Hypophosphorylated Rb is associated with $G_{1/0}$ arrest and its cyclin-dependent phosphorylation in G_1 allows progression from G_1 to S. The loss of phosphorylated Rb by 12-18 h after addition of butyrate, supports the hypothesis that butyrate induced G_1 growth arrest was mediated by hypophosphorylated Rb.

The significance of the role of hypophosphorylated Rb in butyrate-induced apoptosis is not clear. However, other studies have shown that in HL-60 cells treated with arabinoside or etopside for a few hours, hyperphosphorylated Rb decreased while

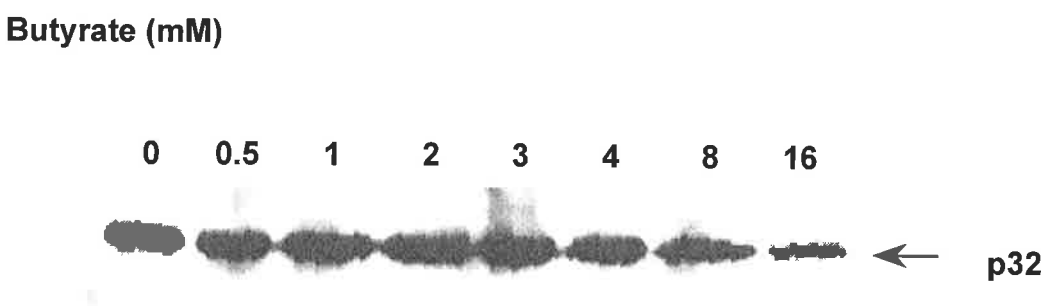


Figure 3.11 Activation of caspase-3 in LIM1215 cells treated by varying concentrations of butyrate. Cells were incubated with varying concentrations of butyrate for 24 h, then harvested for Western Blotting. Equal amounts of protein (36 μg) protein were loaded. This figure shows the activation of caspase-3 as determined by decrease of the precursor form (cpp32) with 16mM butyrate, but not lower concentrations of butyrate (see discussion). The activated products of caspase-3 were not detected by the antibody used in this experiment.

hypophosphorylated Rb increased and these events occurred at an early stage of apoptosis (Dou *et al.*, 1995). Several other research groups have also reported apoptosis-associated Rb dephosphorylation in different cell systems and with different apoptotic stimuli (Morana *et al.*, 1996; Wang *et al.*, 1996). In one study, hypophosphorylation of Rb was followed by its cleavage to p68 and p48 fragments (An and Dou, 1996). In butyrate treated LIM1215 cells, there was an apparent loss of intact Rb forms (Fig 3.6) but the Rb fragments were not detected, probably because of the specificity of the antibody used. Therefore, hypophosphorylation of Rb, followed by its disappearance, may be a common early event in apoptosis. It has previously been shown that hypophosphorylated Rb (pRb) is a substrate for caspase-3 (Dou and An, 1998). The loss of pRb in butyrate-treated cells may be a consequence of caspase-3 activation. In cells treated with 1 mM butyrate, where there is no induction of caspase-3 activity, pRb will accumulate and lead to growth arrest (Fig 3.12A). However, in cells treated with 4 mM butyrate, there is induction of caspase-3 activity, and therefore pRb is cleaved, preventing cells from growth arrest and enabling them to undergo apoptosis (Fig 3.12B).

Butyrate-induced apoptosis involved an increase in activity of DEVD-caspase at concentrations of butyrate (≥ 4 mM). However, the Western blotting study did not show apparent loss of the cpp32 precursor except at the highest concentration of butyrate (16 mM) tested. This discrepancy could be due to another caspase being activated which can also cleave DEVD-containing substrates, for example caspase-7. It was not possible to test this since antibody to caspase-7 was unavailable. Alternatively, it is possible that butyrate may affect the levels of cpp32 by increasing its transcription. This would complicate the interpretation of the Western blot since the intensity of the cpp32 band will then depend

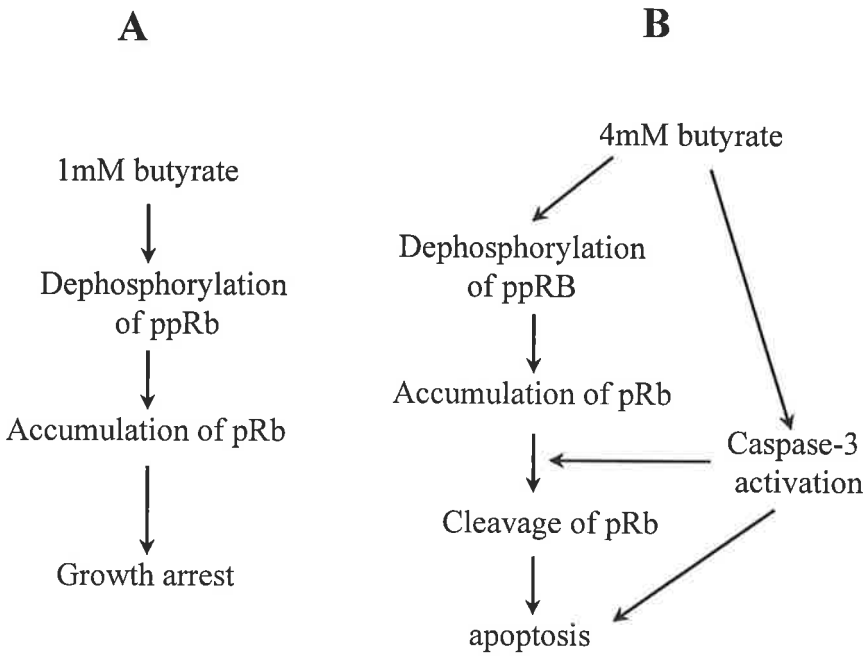


Figure 3.12 Model explaining the relationship between butyrate, pRb and growth arrest/apoptosis.

This model attempts to explain the different effects of 1mM and 4mM butyrate on growth arrest and apoptosis in CRC cells.

A: In cells treated with 1 mM butyrate, there is dephosphorylation of ppRb, resulting in accumulation of pRb and consequent G_1 arrest.

B: In cells treated with 4 mM butyrate, there is dephosphorylation of ppRb. However, there is less accumulation of pRb since 4 mM butyrate also activates caspase-3 which cleaves pRb. As a consequence most of the cells fail to arrest in G_1 but undergo apoptosis.

upon the relative amounts of synthesis and cleavage of cpp32. Future studies should investigate the effects of butyrate on transcription of cpp32, the effects of butyrate on other caspases and the effects on the cleaved subunits of cpp32 using other caspase-3 antibodies to confirm the activation of caspase 3 in these cells.

An interesting finding was that much lower levels of DEVD-caspase activity and DNA fragmentation in SW480 cells compared to LIM1215 cells. Since SW480 cells contain mutant p53, whereas LIM1215 cells contain wild-type p53; it is possible that action of butyrate is, at least partly, p53-dependent. However, others have shown that is not the case. This finding may reflect altered sensitivity/resistance to butyrate in the two cell lines (see chapter 5).

In conclusion, the experiments described in this Chapter have confirmed using several assays that butyrate induces apoptosis of colorectal cancer cell lines and have studied the relationships between caspase activation, apoptosis, Rb dephosphorylation and cell cycle arrest in these cells. Briefly, different concentrations of butyrate induce growth arrest and apoptosis, implying that the mechanism is at least partly different. However, the time-frame of effect on both parallels changes in Rb, and butyrate-induced G_1 arrest is mediated by hypophosphorelated Rb. Higher doses of butyrate result in cleavage of pRb enabling apoptosis. Butyrate activates colorectal cancer cell lines to undergo cleavage of caspase-3 and perhaps other caspases.

Some of these results have been published (Chai *et al.*, 2000).

CHAPTER 4

INVOLVEMENT OF $P21^{WAF1/CIP1}$ IN BUTYRATE-INDUCED APOPTOSIS

4.1 INTRODUCTION

The orderly progression of cells through different cell cycle states is controlled by the sequential activity of various cyclin-dependent kinases (CDKs) which are activated by cyclins and blocked by CDK inhibitors (O'Connor, 1997). There are two major classes of CDK inhibitors, which exert control by binding to cyclin-CDK complexes: the CIP/KIP family, including $p21^{Waf1/Cip1}$, $p27^{Kip1}$ and $p57^{Kip2}$, capable of interacting with most cyclin-CDK complexes, and a second family $p15^{INK4B/MTS2}$, $p16^{INK4/MTS1}$, p18 and p19, with a more restricted range of activity. Increased levels of CIP/KIP proteins are associated with growth arrest and/or differentiation, although the particular protein involved appears to be cell type-dependent. By far the most widely studied of these is p21, a potent inhibitor of a wide array of CDK-cyclin complexes and which is responsible for G_1/S arrest induced by p53 in response to DNA damage (O'Connor, 1997). p21 is also influenced by p53-independent pathways (Nakano *et al.*, 1997).

In addition to its N-terminal CDK inhibitory domain, p21 contains a PCNA binding motif located in the carboxy terminal part of the protein which may be important for the inhibition of DNA replication by p21 (Waga and Stillman, 1998). P21 has been shown to inhibit PCNA-dependent DNA replication and mismatch repair in vitro (Flores Rozas *et al.*, 1994; Umar *et al.*, 1996). PCNA is also required for nucleotide excision repair but p21 does not appear to block the function of PCNA in this process (Flores Rozas *et al.*, 1994; Li *et al.*, 1994; Shivji *et al.*, 1994; Waga *et al.*, 1994).

The interaction of $p21^{Waf1/Cip1}$ with CDK inhibits CDK kinase activity and hinders its ability to phosphorylate the retinoblastoma family of proteins. As discussed in Chapter 3, dephosphorylation of ppRb is associated with butyrate-induced apoptosis. This chapter

concerns the involvement of $p21^{Waf1/Cip1}$ in butyrate-induced apoptosis. From here on, $p21^{Waf1/Cip1}$ will be referred to as p21.

During the course of this thesis, some papers appeared in the literature, suggesting that butyrate was a potent inducer of p21 in CRC cells, even at sub-toxic concentrations (eg 1 mM), and p21 was required for butyrate-mediated growth arrest (Archer *et al.*, 1998; Nakano *et al.*, 1997). However, in NIH3T3 cells, Vaziri et al (1998) reported that growth arrest in the G_1 phase of the cell cycle in response to butyrate was largely independent of p21. Interestingly, p21 is associated with both induction of differentiation (Vaziri *et al.*, 1998) and inhibition of apoptosis (Poluha *et al.*, 1996) in certain systems. Here, the relationship of p21 expression to caspase activation and apoptosis in LIM 1215 CRC cells treated with butyrate was studied, to investigate further the inter-relationships between butyrate induction of apoptosis and cell cycle arrest.

4.2 RESULTS

4.2.1. Regulation of p21Waf1/Cip1 protein and mRNA levels by butyrate

To determine the effect of butyrate on expression of p21, a low concentration (1 mM) of butyrate which induces growth arrest but not apoptosis, and a high concentration (4 mM) which induces apoptosis, were used to treat LIM 1215 colorectal cancer cells. Cells were treated for up to 24 h, and at 3 h interval, cells were harvested and lysates were obtained for p21 expression by Western blotting. Fig 4.1 shows the induction of p21 by 1 mM butyrate. Low basal expression of p21 was detected in untreated control cells (time 0). p21 increased with time especially 12 h after addition of butyrate, and peaked at 15 h.

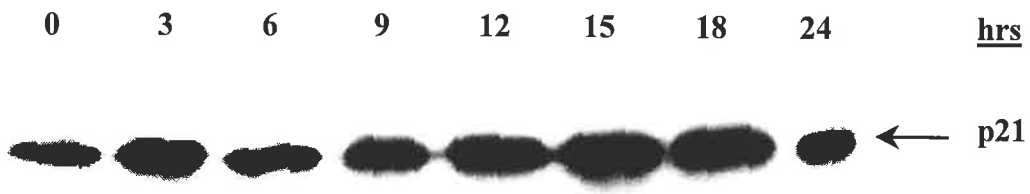


Figure 4.1 Western blot showing induction of p21 by 1 mM butyrate in LIM 1215 cells. Cells were treated with 1 mM butyrate for up to 24 h and at times indicated were harvested for Western blotting. Equal amounts of protein (18 μg) were loaded in each lane. This figure shows that the induction of p21 by butyrate is time dependent, increasing gradually to a peak at 15 h.

The kinetics of p21 induction by 4 mM butyrate is illustrated in Fig 4.2. Increase in p21 protein peaked at 18h after addition of butyrate and was maintained at elevated levels up to, at least, 24 h. The plateau levels of p21 in LIM1215 cells treated with 1 mM or 4 mM butyrate appear to be similar (Fig 4.1, Fig 4.2). At time points beyond 12 h following addition of 4 mM butyrate, a lower molecular weight band, p15, appeared and peaked at 18h. This band is likely to represent a cleavage product of p21.

To investigate further whether butyrate up-regulates p21 expression, butyrate-treated or untreated LIM 1215 cells were assayed for p21 mRNA expression by Northern blot analysis. Fig 4.3 shows the kinetics of expression of p21 mRNA. Basal levels of p21 mRNA are shown at time 0. A substantial increase occurred within 3h after addition of 4 mM butyrate and decreased thereafter, but remained elevated over basal levels up to 18 h. Beyond 18 h, p21 mRNA declined rapidly and was almost undetectable at 30 h. Equal loading of RNA in each track was confirmed by hybridisation of the filters with the GAPDH control probe (Fig 4.3, middle panel). The ratio of p21/GAPDH mRNA is shown as a densitometric profile in the lower panel of Fig 4.3 and confirms the specific induction of p21 mRNA by butyrate.

4.2.2 The appearance of p15 following the activation of DEVD-caspase

As shown above, induction of p21 by butyrate was accompanied by the appearance of a band at molecular weight of about 15kDa. This p15 band appeared at time points beyond 12 h following addition of 4 mM butyrate and peaked at 24 h (Fig 4.2, Fig 4.4A). p15 was consistently seen in Western blots of p21 in butyrate-treated LIM 1215 cells but varied considerably in intensity. Increase in DEVD-caspase activity in butyrate treated LIM 1215 cells occurred between 9 and 12 h after addition of butyrate (closed circles in

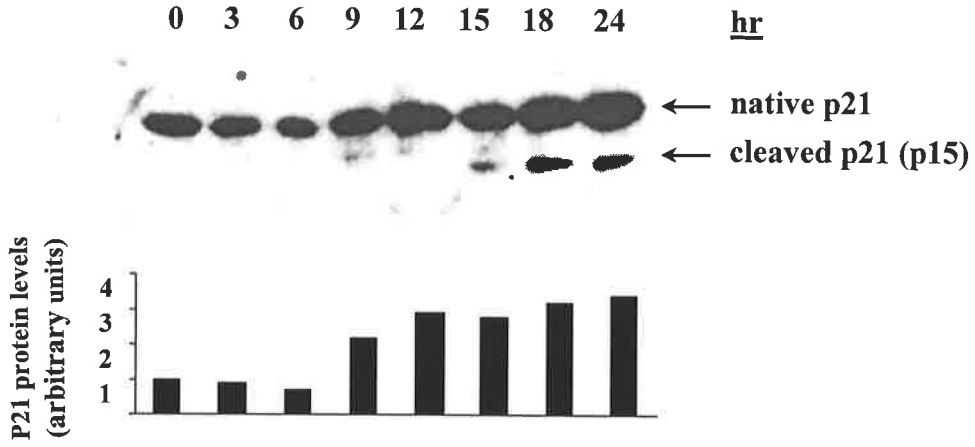


Figure 4.2 Western blot showing induction of p21 and lower molecular fragment p15 by 4 mM butyrate in LIM 1215 cells. The figure shows induction of p21 protein beginning at 9 h followed by appearance of p15 at 15 h after addition of butyrate. Equal amounts of protein (18 μg) were loaded in each lane. A densitometric profile showing the relative levels of p21 protein in each track is shown at the bottom. The results are representative of three independent experiments.

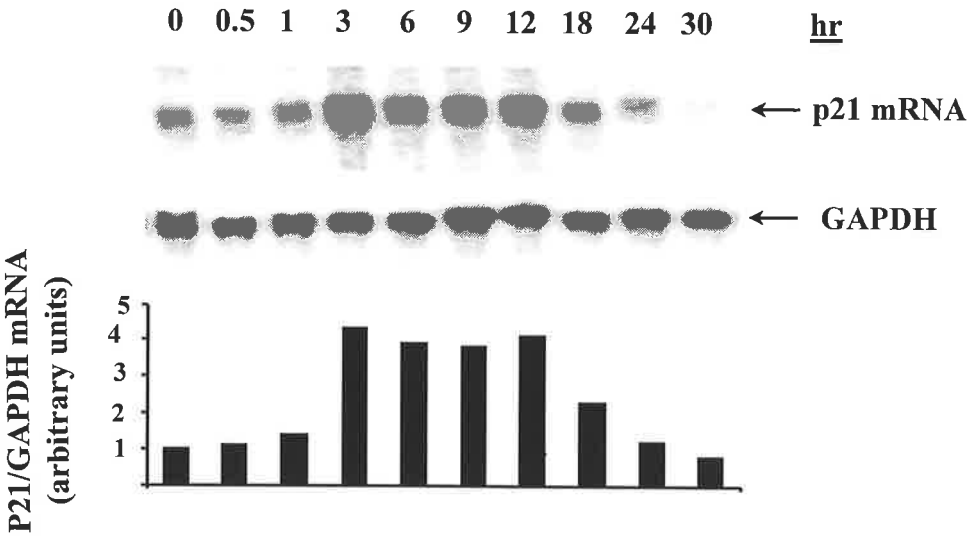


Figure 4.3 Northen blot showing induction of p21 mRNA by 4 mM butyrate in LIM 1215 cells. LIM 1215 cells were treated with or without 4 mM butyrate for the indicated times and p21 mRNA assessed by Northern blotting (top). Each lane contained 10 μg of RNA. The figure shows induction of p21 mRNA 3 h after addition of butyrate; this was maintained at high levels up to 12 h. Blots were rehybridized with a cDNA probe specific for GAPDH to monitor RNA loading (middle). Results were analyzed by densitometry and expressed as a ratio of p21 to GAPDH mRNA (bar chart, lower panel)

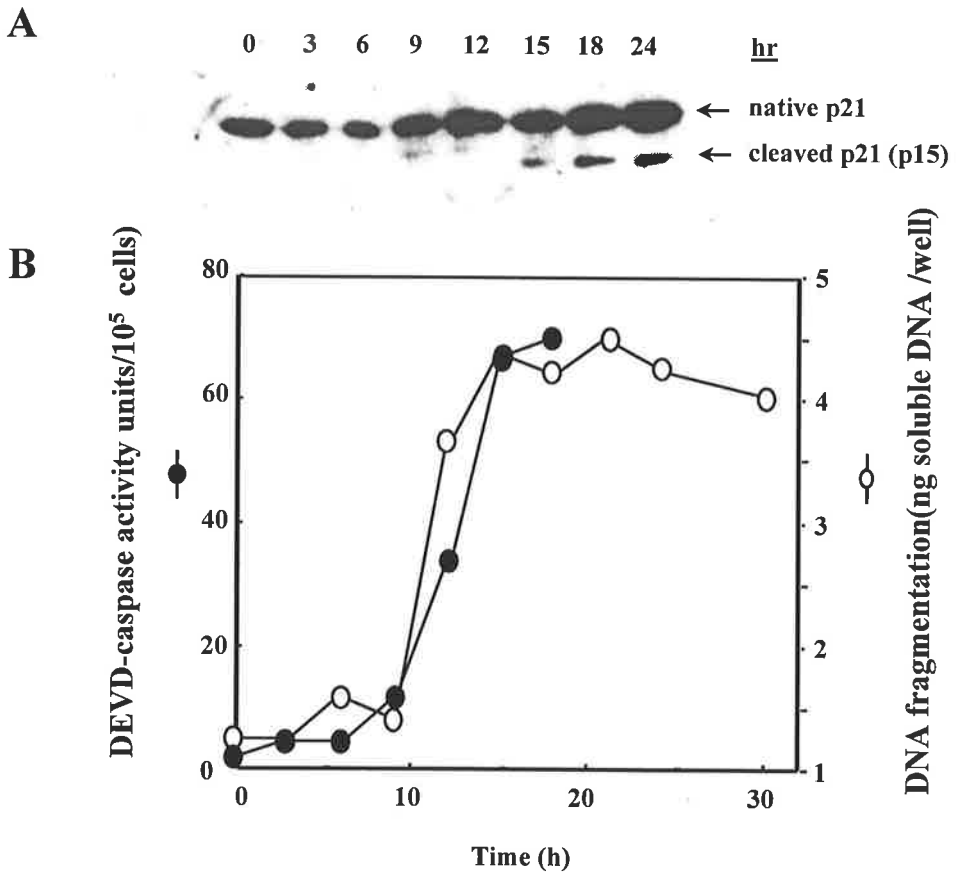


Figure 4.4 Induction of DEVD-caspase activity and DNA fragmentation precedes the cleavage of p21.

A: Western blot of p21 and p15. The figure shows the appearance of p15 at 15 h after addition of 4 mM butyrate. This figure is redrawn from Fig 4.2.

B: Time course of DEVD-caspase activity and DNA fragmentation. This figure shows caspase 3 activity (filled circles) and DNA fragmentation (open circles) increased after 9 h and peaked at 15 h after addition of 4 mM butyrate. This figure is redrawn from Fig 3.10.

Fig 4.4B). This coincided with the induction of DNA fragmentation (open circles in Fig 4.4B), another marker of apoptosis. The first appearance of p15 soon after induction of DEVD-caspase in these cells suggested it may be a consequence of proteolytic cleavage of p21 by DEVD-caspase or another member of the caspase family.

In support of this hypothesis, when lysates of the floating, non-adherent (apoptotic-rich) cell population from 24h butyrate-treated samples were run individually on Western blots, p15 was the predominant fragment (Fig 4.5, tracks c and d). This is in contrast to the situation with combined whole population lysate of butyrate-treated cells, where p21 band is much more abundant than p15 band (Fig 4.5, track b). In the absence of butyrate, no p15 was present (Fig 4.5, track a). This suggests that p21 is entirely cleaved late in apoptosis of colorectal cancer cells. On the other hand, when the lysates of adherent (largely viable or pre-apoptotic) cells were run, both p21 and p15 were detected (Fig 4.5, tracks e and f), implying that p21 is cleaved prior to detachment of the cells from the substratum. Lysates of adherent cells contained significant DEVD-caspase activity (not shown). Note, that despite loading equivalent amounts of protein in tracks c-f, the p15 band in the lysates of the non adherent, largely apoptotic population was much less intense than that of the adherent, largely viable cell population. This may indicate that p15 is degraded further, later in apoptosis

4.2.3 Caspase inhibitors blocked the cleavage of p21

Further evidence for a relationship between p15 and caspase cleavage was sought using peptide caspase inhibitors. LIM 1215 cells were pre-treated with either of two peptide inhibitors, the more specific DEVD-caspase inhibitor zDEVD-fmk or the broad spectrum inhibitor zVAD-fmk (both as fluoryl methyl ketone derivatives). The cells were

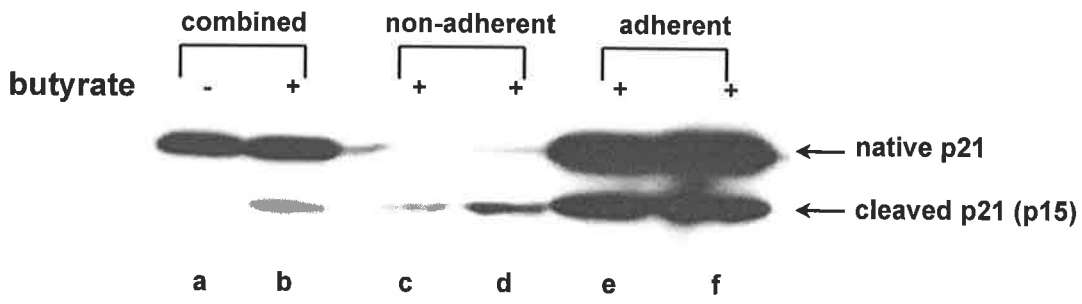


Figure 4.5 Relative amounts of p15 and p21 in adherent and non-adherent cell populations. Cells were treated with or without 4 mM butyrate for 24 h and lysates analyzed by western blotting with anti-p21 antibody. (a) untreated cells (combined non-adherent and adherent populations); (b) butyrate-treated cells (combined non-adherent and adherent populations); (c) butyrate-treated cells (apoptotic cell-rich non-adherent population); (d) duplicate of (c); (e) butyrate-treated cells (adherent population), (f) duplicate of (e). The figure shows enrichment of p15 relative to p21 in apoptotic cells. Note the large decrease in the absolute amount of p21 fragment in non-adherent cells suggesting that once cleaved to p15, it may undergo further rapid degradation.

then treated with butyrate for 24 h and cell lysates analyzed by Western blotting. There was a consistent decrease in p15 with 100 μM zDEVD (compare lane 3 with lane 1 in Fig 4.6A) or 100 μM zVAD (compare lane 5 with lane 1 in Fig 4.6A). Suppression of p15 was more pronounced with zVAD-fmk and the band completely disappeared at a zVAD-fmk concentration of 100 μM (lane 5). zDEVD-fmk gave only partial inhibition at concentrations up to 100 μM (the highest concentration tested, lane 3). Cells treated with either zDEVD alone (lane 6), zVAD alone (lane 7), or control cells untreated with butyrate (lane 8) showed no cleavage product. DEVD-caspase activity in the cells was more than 90% suppressed at concentrations of 0.6 μM and greater for zDEVD-fmk (open circles in Fig 4.6B) and 5 μM and greater for zVAD-fmk (filled circles in Fig 4.6B). However, inhibition of p21 cleavage was only partial, suggesting that p21 may also be cleaved by other proteases.

4.2.4 Caspase-3 cleavage motif in primary sequence of p21

Subsequent analysis of the p21 amino acid sequence revealed a likely caspase-cleavage motif DHVD between amino acids 109 and 112 (Fig 4.7A, Fig 4.8). Cleavage after D_{112} would yield fragments with predicted M_r of 12.4 KDa and 6.0 KDa. The larger of these would be consistent with the size of the p15 fragment, since whole p21 has a calculated M_r of 18.4 KDa but migrates on SDS-PAGE with an apparent M_r of 21 KDa. It should be noted that the corresponding sequence for feline p21 contains DHLD (also a potential caspase-cleavage motif) but mouse and rat p21 contain DHVA (Fig 4.7) which can not be cleaved by caspase-3, since the terminal D (aspartate) is essential.

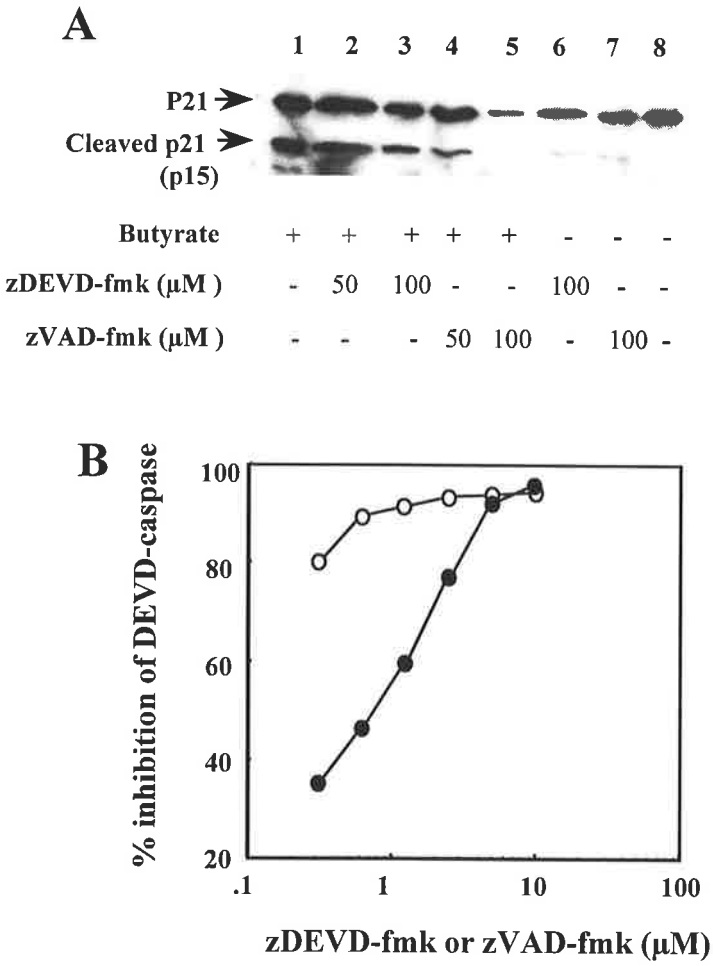


Figure 4.6 Effect of caspase peptide inhibitors on p21 cleavage and DEVD-caspase activity in butyrate-treated LIM 1215 cells.

(A) Effect of inhibitors on p15 determined by Western blotting. Cells were treated with 4 mM butyrate for 24 h in the presence of the indicated concentrations of zDEVD-fmk or zVAD-fmk before assaying p15 by Western blotting. The figure shows partial suppression of p21 cleavage by 100 μM zDEVD-fmk and complete suppression by 100 μM zVAD-fmk.

(B) Effect of inhibitors on butyrate-induced DEVD-caspase activity in LIM 1215 cells. zDEVD-fmk (open circles); zVAD-fmk (closed circles). The figure shows almost complete suppression of cellular caspase activity by both inhibitors at much lower concentrations than required to suppress p21 cleavage. These results are representative of three independent experiments.

A: human

```
  1 msepagdvrq npcgskacrr lfgpvdseql srdcdalmag ciqearerwn fdfvtetple
 61 gdfawervrg lglpklylpt gprrgrdelg ggrrpgtspa llqgtaeedh vdlslsctlv
121 prsgeqaegs pggpgdsqgr krrqtsmtdf yhskrrlifs krkp
```

B: murine

```
  1 msnpgdvrpv phrskvcrcl fgpvdseqlr rdcdalmagc lqearerwnf dfvtetpleg
 61 nfvwervrsl glpkvylspg srsrddlggd krpstssall qgpapedhva lslsctlvse
121 rpedspggpg tsqgrkrrqt sltdfyhskr rlvfckrkp
```

Fig 4.7 Primary sequence of p21. Figure shows the amino acid sequences of (A) human p21 (obtained from Harper, et al., 1993) and (B) murine p21 (from El-Deiry, et al. 1995). Note the consensus motif DHVD for caspase-3 shown in bold in human p21 but altered to the non-cleavable motif DHVA in murine p21 .

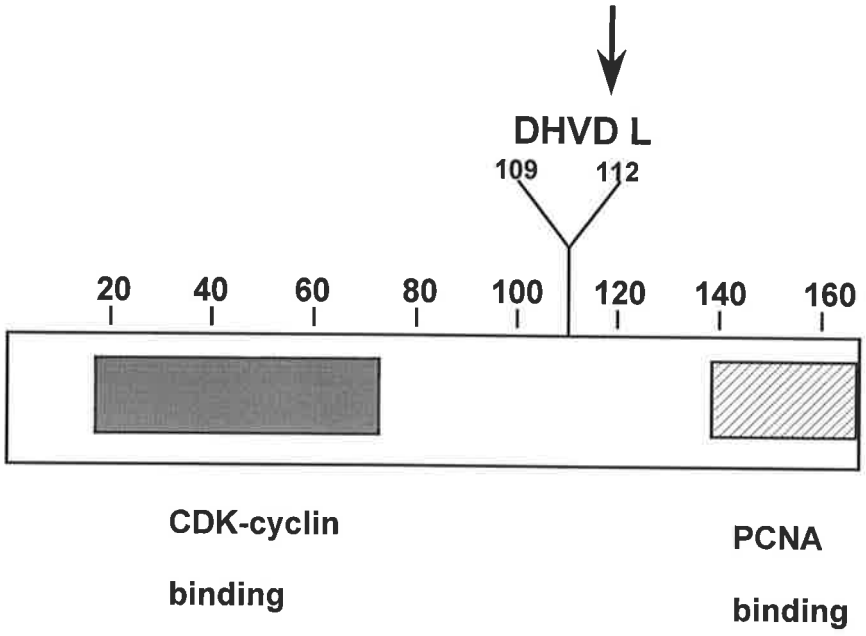


Figure 4.8 Structure of p21 showing the position of the caspase cleavage motif. The large grey rectangle depicts the 164 amino acid p21 protein with the N-terminal CDK-binding site (filled rectangle) and the C-terminal PCNA-binding site (hatched rectangle). The potential caspase cleavage motif DHVD occurs between these sites at positions 109–112. The arrow shows the proposed site of cleavage.

4.3 DISCUSSION

The aim of these studies was to explore further the mechanism by which butyrate regulates apoptosis and cell cycle arrest of colorectal cancer cells, in vitro. It is thought that butyrate induces apoptosis by a novel pathway initiated by inhibition of histone deacetylase and resulting in expression of a putative cell death gene which acts synergistically with the Bcl-2-regulated mitochondrial/cytochrome c-mediated pathway (Medina *et al.*, 1997). These two pathways converge at a point upstream of caspase-3 protease, a major effector of apoptosis. This chapter shows that induction of p21, and its cleavage, parallel in time these effects and may be critical regulatory events.

The induction of p21 by butyrate in LIM 1215 cells confirms studies in other colorectal cancer cell lines (Waga and Stillman, 1998; Nakano *et al.*, 1997) and in other types of cell (Gartel *et al.*, 1996; Archer *et al.*, 1998). LIM 1215 cells have wild-type p53 (Agapova *et al.*, 1996) and are likely to exhibit p53-dependent up-regulation of p21 in response to some stimuli eg DNA-damaging agents. Further induction of p21 by butyrate may be a direct effect mediated by a butyrate-responsive element in the promoter region of the p21 gene (Nakano *et al.*, 1997). The rapid decline in p21mRNA after 12 h was not matched by a similar decline in p21 protein, suggesting that p21 does not rapidly turn over in LIM1215 cells. p21 was induced during the interval of 3 to 12 h, previously defined by cycloheximide inhibition studies for expression of the essential pro-apoptotic protein (Medina *et al.*, 1997). This raises the question of whether p21 is this protein or whether it is increased as a cellular protective mechanism against apoptosis. Appearance of p21 immediately preceded the appearance of cytosolic DEVD-caspase activity and onset of DNA fragmentation. However, it seems unlikely that the butyrate-inducible pro-apoptotic

protein is p21 since several studies have shown that p21 is anti-apoptotic. For example, anti-sense oligonucleotides which block p21 expression facilitate, rather than suppress, apoptosis in neuroblastoma cells (Teare *et al.*, 1997). It is possible that by maintaining the cells in G_1, p21 prevents critical events leading to caspase activation.

The detection of an apparent fragment of p21 (designated p15) at later time points in butyrate-treated LIM 1215 cells was a surprising finding. Subsequently, several reports appeared in the literature showing p21 cleavage in other types of cells undergoing apoptosis in response to various stimuli. These include cleavage of p21 in tumour-necrosis factor-induced apoptosis of human cervical carcinoma cells (Donato and Perez, 1998), in growth factor-deprived human endothelial cells (Levkau *et al.*, 1998), and in response to DNA damage by gamma-irradiation and DNA damaging agents (Gervais *et al.*, 1998; Zhang *et al.*, 1999). In support of the hypothesis that the p15 fragment seen here after butyrate treatment is a caspase cleavage product of p21, are the following observations: (a) p15 began to appear soon after DEVD-caspase was activated, (b) its appearance coincided with apoptosis in a butyrate concentration-dependent manner, (c) it was greatly enriched in the floating apoptotic cells, and (d) its appearance was partly suppressed by peptide caspase inhibitors.

Following observation of the p15 fragment, a search of the sequence of amino acids in p21 revealed a potential caspase-3 cleavage motif DHVD between amino acids 109 and 112. Cleavage after amino acid 112 would generate a fragment consistent with the size of p15. This motif was also pointed out by Levkau and colleagues (Levkau *et al.*, 1998), who confirmed that p21 was a caspase substrate by site-directed mutagenesis. The latter study also showed cleavage of $p27^{Kip1}$ in growth factor-deprived endothelial cells.

The cleavage of p21 by caspases mimics the cleavage by these proteases of certain other signal transduction proteins (Widmann *et al.*, 1998). However, it should be noted that p21 may also be cleaved via a proteasome-dependent mechanism (Blagosklonny *et al.*, 1996) which may account for only partial suppression of cleavage of p21 by zDEVD-fmk in butyrate-treated LIM 1215 cells, despite complete suppression of DEVD-caspase activity. This may indicate that other caspases and/or other proteases are involved in the cleavage of p21. The presence of a unique caspase-specific motif DHVD in p21 suggests that cleavage of p21 is not simply reflecting wide-scale proteolytic degradation.

Disruption of cell-cycle regulatory check-points may be essential before suicide mechanisms can be turned on. In the absence of p21, DNA-damaged colon cancer cells undergo repeated S-phases, without intervening mitoses, before dying by apoptosis (Waldman *et al.*, 1997). However, the rapidity of butyrate-induced apoptosis excludes this mechanism for killing of LIM 1215 cells by this agent. The experiments in this chapter suggest that cleavage of p21 in colorectal cancer cells follows caspase activation but precedes detachment of cells from substratum. This event might be important for apoptosis in either of two ways. Apoptosis may be allowed to proceed as a result of inactivation of p21 following cleavage, thereby disrupting its anti-apoptotic action or, alternatively, the cleavage products themselves might actively promote apoptosis (Fig 4.9). Relevant to this, Prabhu et al, (1996) showed that over-expression in cancer cells of a p21 truncation mutant, derived by introduction of a premature stop codon, induced apoptosis of the cells whereas the wild type p21 only induced growth arrest. Coincidentally, the truncation mutant used in those studies terminated at L_{113}, one amino

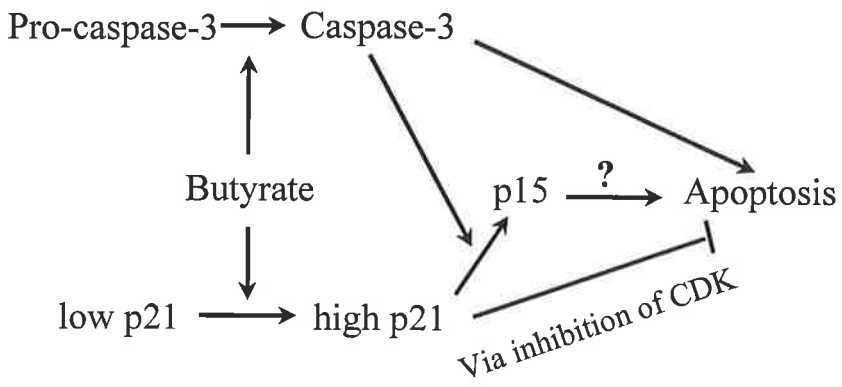


Figure 4.9 Interaction between caspase-3, p21 and its cleavage product p15 and apoptosis in butyrate-treated LIM1215 cells. Butyrate up-regulated p21 and activated caspase-3 which cleaved p21 to p15, disrupting the anti-apoptotic action of p21. P15 may promote apoptosis.

acid beyond the putative DHVD caspase-cleavage site. Therefore, induction of apoptosis by over-expression of the caspase-derived fragment seems the more likely mechanism.

p21 is a polypeptide of 164 amino acids with a CDK-binding site in the N-terminal portion and a PCNA binding site in the C-terminal portion (Fig 4.8) (O'Connor, 1997). The significance of the PCNA binding is unclear. Recently, Lu and colleagues (Lu *et al.*, 1998) showed that mutant p21, lacking CDK-inhibitory activity but still retaining PCNA-binding, no longer protected a colorectal cancer cell line from induction of apoptosis by X-rays or adriamycin, implying that inhibition of CDK is critical to p21-mediated suppression of apoptosis (Fig 4.9). In another study, mutants lacking PCNA-binding were unable to arrest growth, although apoptosis was not investigated (Cayrol *et al.*, 1998). Since the putative DHVD cleavage motif lies between the CDK-cyclin- and PCNA-binding domains, cleavage may disrupt the function and/or subcellular localization of p21. One possibility is that p15, which still retains the CDK-cyclin binding domain, competes with remaining p21 for CDK but is unable to inhibit it because PCNA is no longer in the complex. Alternatively, p15 may fail to localize in the nucleus because it lacks the PCNA binding site.

Of interest, p21 from mouse and rat lack the critical aspartate residue preceding the leucine at the putative caspase-3 cleavage motif. It is known that replacement of this second D by A in caspase cleavage motifs by site–directed mutagenesis abolishes caspase cleavage of substrates (Kook *et al.*, 2000; Thornberry *et al.*, 1997). Therefore, p21 is unlikely to be a caspase substrate in these other species. However, feline p21 did contain a caspase-3 motif. The significance of these species-specific differences for regulation of apoptosis is not clear. Further studies should investigate whether p15 is formed during butyrate–induced apoptosis of mouse and rat CRC or other cell lines.

Therefore, expression of p21 and its cleavage are likely to have important implications, not only for colorectal tumorigenesis but also for mechanisms of regulation of cell turnover in normal intestinal epithelium. Expression of p21 marks the transition from proliferation to terminal differentiation in intestinal epithelium (Gartel *et al.*, 1996). Cleavage of p21 may, in turn, mark the transition from terminal differentiation to apoptosis at the luminal ends of the crypts. The importance of caspase-mediated turnover of p21 versus that of transcriptional regulation now needs to be defined. It is possible that cleavage of p21 is a novel regulatory mechanism that facilitates apoptotic death of colon cancer cells by butyrate and perhaps other stimuli.

Some of the experiments described in this chapter are in the published article (Chai *et al.*, 2000).

CHAPTER 5

MECHANISMS INVOLVED IN RESISTANCE TO BUTYRATE-INDUCED APOPTOSIS

5.1 INTRODUCTION

Historically, butyrate was recognised as a differentiation-inducing agent for colorectal cancer cell lines. Differentiation was evident in these studies from the changes in the following properties: increase in differentiation markers such as alkaline phosphatase, carcinoembryonic antigen (CEA), urokinase receptor and plasminogen activator inhibitor-1, increase in transepithelial resistance and morphological changes (Mariadason *et al.*, 2000). However, it is not clear whether this is a real, irreversible differentiation to an end stage or rather a transient phenotypic change that can be reversed by withdrawal of butyrate. The early studies did not recognise the apoptosis-inducing effects of butyrate, probably for two reasons: butyrate was used at sub-toxic concentration (e.g. 1 mM) and any apoptotic cells, which were largely floating in the medium, were discarded prior to harvest of the remaining adherent cells for differentiation marker studies. It is now clear that butyrate can induce both differentiation and apoptosis in the same population of cells, but perhaps by multiple pathways (Mariadason *et al.*, 2000).

The experiments in chapter 3 showed that butyrate induced apoptosis in LIM1215 and SW480 cells by a number of criteria, including morphological changes, flow cytometric change in propidium iodide fluorescence, DNA fragmentation and DEVD-caspase/caspase-3 activation. However, not all of the cells underwent apoptosis. For example, following treatment with an optimal concentration (4mM) of butyrate for 24 h, 50%–60% of the cells apoptosed, as determined by cell viability and flow cytometric assays. No further apoptosis occurred if the incubation time with butyrate was extended. In addition, both DNA fragmentation and DEVD-caspase activity peaked at 15-18 h and did not increase with further time. There are several possible reasons for the survival of

the remaining 40%-50% of cells: 1) these cells may metabolise butyrate as a fuel; it is possible that butyrate is a limiting factor in these cultures and that if further butyrate was added at 24 h then more of the cells would have died; 2) these cells may undergo differentiation and no longer be capable of apoptosis; 3) these remaining cells may be resistant to the apoptosis-inducing effects of butyrate.

5.2 RESULTS

5.2.1 The development of butyrate resistance in LIM1215 cells and SW480 cells

Exposure of LIM1215 cells to butyrate resulted in death and detachment of only a proportion of the cells. One possible explanation is that butyrate is limiting; another possibility is that the remaining cells are resistant to butyrate. To test whether those cells remaining attached are resistant to butyrate, cells remaining attached (and viable) after 24 h with butyrate were subjected to a second and third cycle of treatment with butyrate. LIM1215 cells were treated for 3 cycles of 24 h with 1 or 4 mM butyrate. Each cycle consisted of 24 h of treatment with butyrate followed by removal of floating dead cells, prior to further addition of butyrate.

Fig 5.1 shows that 3 cycles of 1mM butyrate treatment induces partial butyrate resistance (middle set of columns). After 3 cycles of 1mM butyrate treatment, DEVD-caspase activity induced by 4 mM and 8 mM butyrate was still substantial, although it was smaller than in butyrate-sensitive cells (left set of columns). However, 3 cycles of 4 mM butyrate treatment induced almost complete butyrate resistance (right set of columns). After 3 cycles of 4 mM butyrate treatment, DEVD-caspase activity induced by 4 mM and

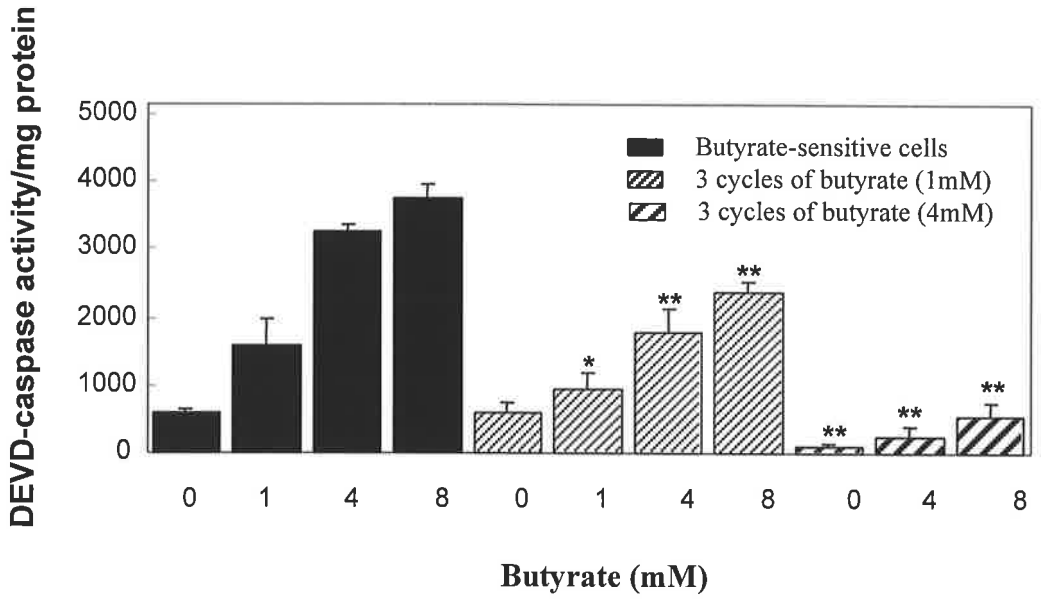


Figure 5.1 Induction of butyrate resistance in LIM 1215 cells. For butyrate-sensitive cells (left set of columns), LIM1215 cells, that had not previously been exposed to butyrate, were treated with indicated concentrations of butyrate for 24 h and then lysates were prepared and assayed for DEVD-caspase. For induction of butyrate resistance, cells were treated with 1mM butyrate (middle set of columns) or 4mM butyrate (right set of columns) for 3 cycles of 24 h, discarding any floating apoptotic cells at the end of each cycle. After the third cycle, cells were treated for a further 24 h with indicated concentrations of butyrate, lysates were prepared and assayed for DEVD-caspase. Figure shows partial induction of butyrate resistance following 3 cycles of 1mM butyrate and almost complete resistance following 3 cycles of 4mM butyrate. Error bars indicate standard deviations for means of triplicates.

* indicates $p \leq 0.05$, ** $p \leq 0.005$. In this figure, the values for DEVD-caspase activity in butyrate resistant cells (middle and right hand sets of columns) were compared with those for the corresponding concentration of butyrate in the butyrate sensitive cells (left hand set of columns).

8 mM butyrate was very markedly reduced. These results suggested that butyrate-induced resistance was concentration-dependent.

Fig 5.2 shows the result of a typical experiment (repeated four times) in which total DEVD-caspase activity (expressed per 10^5 cells) from both the adherent and floating cell populations was assayed. The initial level of DEVD-caspase activity was 3.0 ± 0.3 units of caspase/10^5 cells. The cells were then incubated for 1 day with 4 mM butyrate. There was substantial increase in DEVD-caspase activity (60.6 ± 5.5 units/10^5 cells) as shown in column Ad1. In the absence of butyrate, there were no changes in the levels of DEVD-caspase activity (2.7 ± 0.8 units/10^5 cells). After this first day, the floating cells in each well were decanted and the remaining adherent cells were cultured for a further 24 h with a second application of butyrate (4 mM). Results for this second addition of butyrate are shown in column marked Ad2. In the presence of butyrate, there was still high activity of DEVD-caspase (38.7 ± 2.8 units/10^5 cells), although less than after the first day. The floating cells were again discarded and the remaining cells exposed to a third cycle of butyrate (Ad3). At this stage, there was negligible activation of DEVD-caspase (5.8 ± 0.8 units/10^5 cells). Therefore, in cultures exposed to 3 cycles of butyrate, there was induction of butyrate-resistant LIM 1215 cells.

To determine whether the resistance to butyrate was permanent, the cells were cultured in the absence of butyrate for 2 days. During this withdrawal period (columns W1 and W2 in Fig 5.2), there was no significant DEVD-caspase activation over that in control cells. When butyrate was added after the withdrawal period (Ad1'), there was substantial activation of DEVD-caspase (58.2 ± 6.7 units/10^5 cells), similar to that obtained in the initial treatment with butyrate (Ad1), suggesting complete restoration of sensitivity to

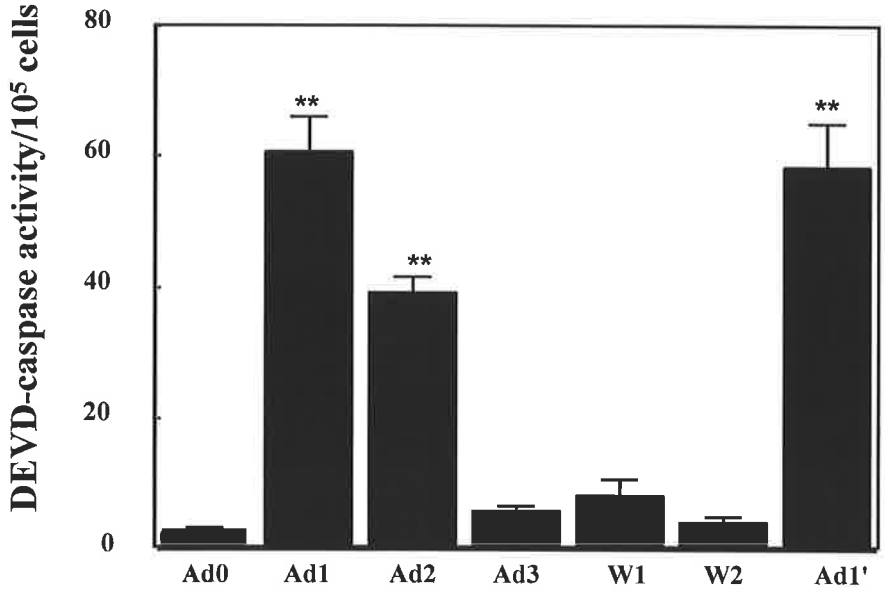


Figure 5.2 Reversibility of butyrate resistance in LIM 1215 cells. The following treatments were performed. After these treatments, the cells were lysed and assayed for DEVD-caspase activity (expressed per 10^5 cells).

Ad0: Cells were untreated.

Ad1: Cells were treated for 24 h with 4 mM butyrate.

Ad2: After Ad1, floating dead cells were removed and 4 mM butyrate added to the remaining adherent cells for a further 24 h.

Ad3: After Ad2, floating dead cells were removed and 4 mM butyrate added to the remaining adherent cells for a further 24 h.

W1: After Ad3, floating dead cells were removed and the remaining adherent cells were cultured in the absence of butyrate for a further 24 h.

W2: After W1, floating dead cells were removed and the remaining adherent cells were cultured in the absence of butyrate for a further 24 h.

Ad1': After W2, floating dead cells were removed and 4 mM butyrate added to the remaining adherent cells for a further 24 h.

The figure shows that DEVD-caspase activity increases markedly on the first day of treatment with butyrate (Ad1) but is sequentially lost following three cycles of addition of butyrate. By day 3 (Ad3), the cells were resistant to butyrate-induced caspase activation. Resistance was reversed when cells were grown in the absence of butyrate for 2 days (W1 and W2), which resulted in sensitive cells when butyrate was added again following this withdrawal period (Ad1'). Error bars indicates standard deviations for means of triplicates.

* indicates $p \leq 0.05$, ** $p \leq 0.005$. In this figure, the values for DEVD-caspase activity were all compared with that of Ad0 cells.

butyrate. Therefore, the resistance to butyrate in cells at the Ad3 stage was only temporary and disappeared when cells were cultured in the absence of butyrate for 2 days. Subsequent experiments showed that in order to reverse the resistance to butyrate at the Ad3 stage, butyrate had to be withdrawn for at least 2 days (data not shown).

To further confirm this butyrate resistance in another cell line, SW480 cells were treated with 4 mM butyrate following a similar protocol. Although the first 24 h of treatment with 4 mM butyrate induced DEVD-caspase activity (Ad1, Fig 5.3), maximal DEVD-caspase occurred in the second cycle of butyrate treatment (Ad2). The reason for this further increase of DEVD-caspase activity is unclear (see discussion). However, as for LIM1215 cells, the third cycle of butyrate treatment (Ad3) resulted in a significant decrease of DEVD-caspase activity. There was little change when these cells were further incubated with butyrate, even up to the sixth cycle (Ad6, Fig 5.3). These results demonstrate the development of butyrate resistance in SW480 cells.

5.2.2 Cell cycle analysis of butyrate resistant LIM 1215 cells

To further characterise butyrate resistant cells, cell cycle analysis was performed in butyrate-resistant LIM1215 cells. The initial population (Ad0) of LIM1215 cells contained 4.7% apoptotic cells, 57.9% G_1 cells, 23.5% S phase cells and 11.7% G_2+M cells (Table 5.1). After addition of 4 mM butyrate for 24 h (Ad1), apoptosis increased to 50.4% of cells and there were decreases in the other cell cycle phases. After 4 cycles of butyrate treatment (Ad4), the remaining cells were almost completely resistant to butyrate (1.5% apoptotic cells). At this stage, the proportion of cells in each phase of the cell cycle was a little different from that of the initial butyrate-sensitive cell population (Ad0). Ad0 and

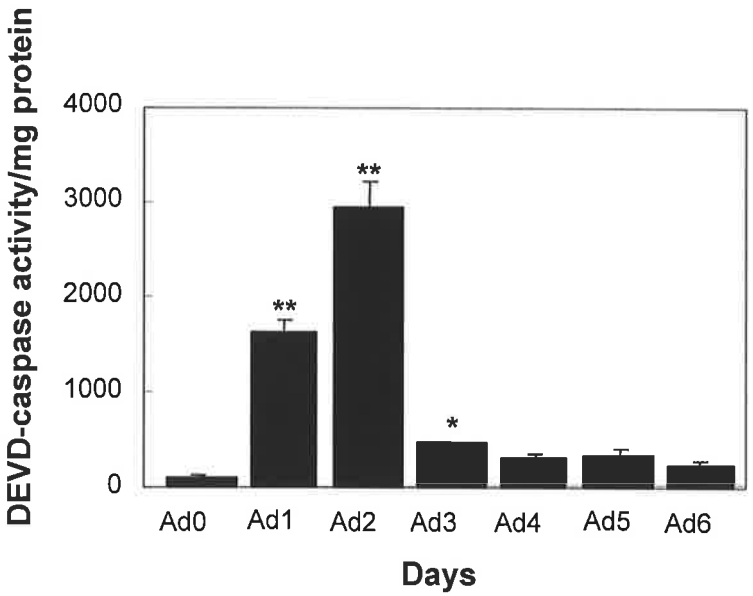


Figure 5.3 Induction of butyrate resistance in SW480 cells. The following treatments were performed. After these treatments, the cells were lysed and assayed for DEVD-caspase activity (expressed per mg protein).

Ad0: Cells were untreated.

Ad1: Cells were treated for 24 h with 4 mM butyrate.

Ad2: After Ad1, floating dead cells were removed and 4 mM butyrate added to the remaining adherent cells for a further 24 h.

Ad3: After Ad2, floating dead cells were removed and 4 mM butyrate added to the remaining adherent cells for a further 24 h.

Ad4: After Ad3, floating dead cells were removed and 4 mM butyrate added to the remaining adherent cells for a further 24 h.

Ad5: After Ad4, floating dead cells were removed and 4 mM butyrate added to the remaining adherent cells for a further 24 h.

Ad6: After Ad5, floating dead cells were removed and 4 mM butyrate added to the remaining adherent cells for a further 24 h.

The figure shows that DEVD-caspase activity increases markedly on the first two days of treatment with butyrate (Ad1 and Ad2) but is lost following three cycles of addition of butyrate (Ad3) and remains low for further additions of butyrate (Ad4, Ad5 and Ad6).

* indicates $p \leq 0.05$, ** $p \leq 0.005$. In this figure, the values for DEVD-caspase activity were all compared with that of Ad0 cells.

Table 5.1 Cell cycle analysis of butyrate resistant LIM1215 cells

% of cells[+]	Adherent cell population[++]			
	Ad0	**Ad1**	**Ad3**	**Ad4**
Apoptotic	4.7	50.4	20	1.5
G_1	57.9	33.5	54.8	61.4
S	23.5	8.2	5.3	12.7
G_2+M	11.7	5.9	18.2	21.9

[+] Percentage of cells that were apoptotic (sub-G1) or in G_1, S, or G_2+M phase of cell cycle was determined by flow cytometry.

[++]Cells populations: **Ad0**: Cells were untreated. **Ad1**: Cells were treated for 24 h with 4 mM butyrate. **Ad2**: after Ad1, floating dead cells were removed and 4 mM butyrate added to the remaining adherent cells for a further 24 h. **Ad3**: after Ad2, floating dead cells were removed and 4 mM butyrate added to the remaining adherent cells for a further 24 h. **Ad4**: after Ad3, floating dead cells were removed and 4 mM butyrate added to the remaining adherent cells for a further 24 h.

Ad4 cells contained 57.9% and 61.4% G_1 phase cells, respectively; they contained 23.5% and 12.7% S phase cells, respectively, and 11.7% as compared to 21.9% G_2+M phase cells, respectively (Table 5.1). The increase in G_2+M phase cells suggests that some of the butyrate resistant cells are in G_2+M arrest.

5.2.3 Butyrate-resistant cells are sensitive to staurosporine but not trichostatin A.

Two possibilities for butyrate resistance are 1) that butyrate resistant cells rapidly metabolise butyrate and 2) that butyrate resistant cells have some alteration in their histone deacetylase, such that it is no longer capable of being inhibited by butyrate. To explore this further, another histone deacetylase inhibitor trichostatin A (TSA) was used. Unlike butyrate, TSA is not metabolised by cells and it has a completely different structure (see Fig 1.3), suggesting that its mechanism of inhibition of histone deacetylase is different from that of butyrate. Therefore, it was important to determine whether butyrate resistant cells were cross-resistant to TSA also. To test this, butyrate resistant LIM1215 cells were incubated with TSA at a concentration (1 μM) known to induce apoptosis in LIM1215 cells (Medina *et al.*, 1997). Fig 5.4 shows that butyrate-sensitive cells (left hand set of columns) were also sensitive to TSA. In fact, at the concentration used, TSA give a much larger increase in DEVD-caspase activity than butyrate and there was no further increase when cells were treated with both butyrate and TSA. The higher response to TSA when compared to butyrate in butyrate-sensitive cells might be a consequence of its failure to be metabolised by cells although other reasons are possible. The right hand set of columns in Fig 5.4 show that butyrate resistant cells are cross-resistant to TSA. While in sensitive LIM1215 cells, TSA induced a 35.8-fold increase in DEVD-caspase activity over the corresponding control, in resistant LIM1215 cells, TSA only induced a 3.8-fold increase

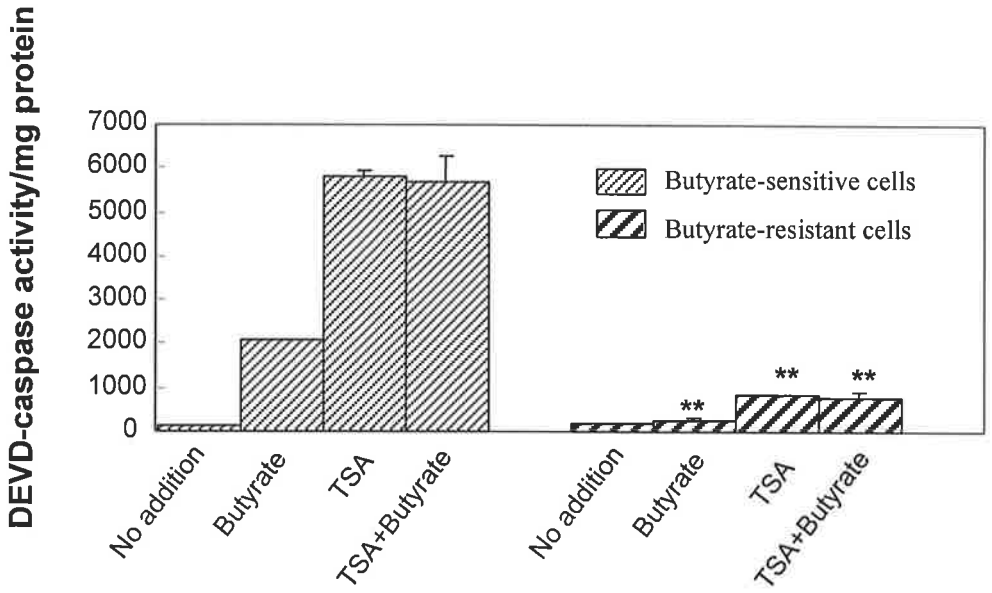


Figure 5.4 DEVD-caspase activity induced by TSA in butyrate sensitive and resistant LIM1215 cells. Butyrate sensitive cells (▨) or butyrate resistant cells (◪) prepared by 3 cycles of addition of butyrate as in Fig 5.1, were treated for a further 24 h with no addition, 4mM butyrate,1 μM TSA or combination of butyrate and TSA. Cell lysates were prepared and assayed for DEVD-caspase activity. Figure shows that butyrate resistant cells are resistant to TSA and therefore still resistant to another inhibitor of histone deacetylase. Error bars indicate standard deviations for means of triplicates.

* indicates $p \leq 0.05$, ** $p \leq 0.005$. In this figure, the values for DEVD-caspase activity in butyrate resistant cells (right hand sets of columns) were compared with those for the corresponding treatments in the butyrate sensitive cells (left hand set of columns).

over the corresponding control. Therefore, rapid metabolism of butyrate can not explain the resistance of cells to butyrate.

To determine whether butyrate resistant cells are also resistant to other apoptosis-inducing agents, staurosporine (STS) was used. In contrast to TSA, STS not only induced apoptosis in butyrate resistant cells, but also potentiated butyrate induced DEVD-caspase activity in these cells (Fig 5.5, right hand columns). Therefore, butyrate resistant cells remain sensitive to another inducer of apoptosis.

5.2.4 p21 is one factor responsible for resistance to butyrate

One possibility was that resistance to the butyrate-induced apoptosis was determined by a cellular protein whose expression was regulated by butyrate. Since butyrate up-regulates p21 (chapter 4), induction of p21 was determined by western blotting in butyrate resistant cells. Comparing Fig 5.2 and Fig 5.6 shows a correlation between the levels of p21 protein and resistance to butyrate-induced apoptosis. Initially, the cells had moderate levels of p21 protein (lane Ad0 in Fig 5.6) and minimal DEVD-caspase activity (Fig 5.2). After addition of butyrate for 1 day (lane Ad1 in Fig 5.6), there was a modest increase in p21 protein, in addition to a lower molecular weight band, p15, which is likely to be a cleavage product (see Chapter 4). These Ad1 cells had high DEVD-caspase activity (Fig 5.2). After three cycles of addition of 4 mM butyrate and removal of floating cells, the remaining adherent cells, which were resistant to butyrate-induced apoptosis (Fig 5.2), had very high levels of p21 protein; the p15 band was no longer detectable (lane Ad3 in Fig 5.6). When butyrate was withdrawn, there was a decline in levels of p21 (lanes W1 and W2 in Fig 5.6). It is interesting to note that the decline in p21 preceded reversal of resistance since at day 1 of withdrawal (lane W1), the

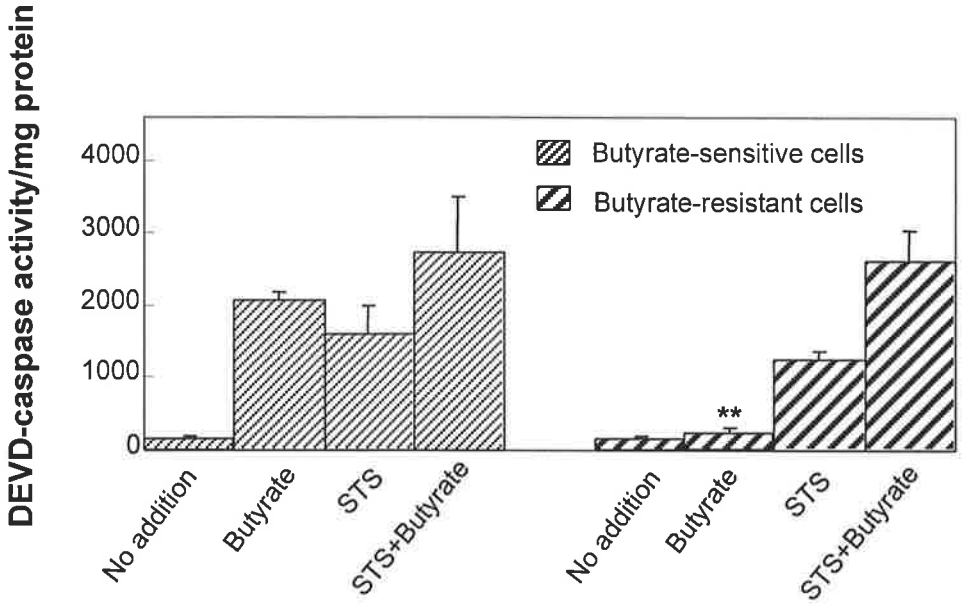


Figure 5.5 DEVD-caspase activity induced by STS (staurosporine) in butyrate sensitive and resistant LIM1215 cells. Butyrate sensitive cells (▨) or butyrate resistant cells (◪) prepared by 3 cycles of addition of butyrate as in Fig 5.4, were treated for a further 24 h with no addition, 4 mM butyrate,1 μM STS or combination of butyrate and STS. Cell lysates were prepared and assayed for DEVD-caspase activity. Figure shows that butyrate resistant cells are sensitive to STS and therefore not generally resistant to apoptosis. Therefore, butyrate resistance affects only some pathways of apoptosis. Error bars indicate standard deviations for means of triplicates.

* indicates $p \leq 0.05$, ** $p \leq 0.005$. In this figure, the values for DEVD-caspase activity in butyrate resistant cells (right hand sets of columns) were compared with those for the corresponding treatments in the butyrate sensitive cells (left hand set of columns).

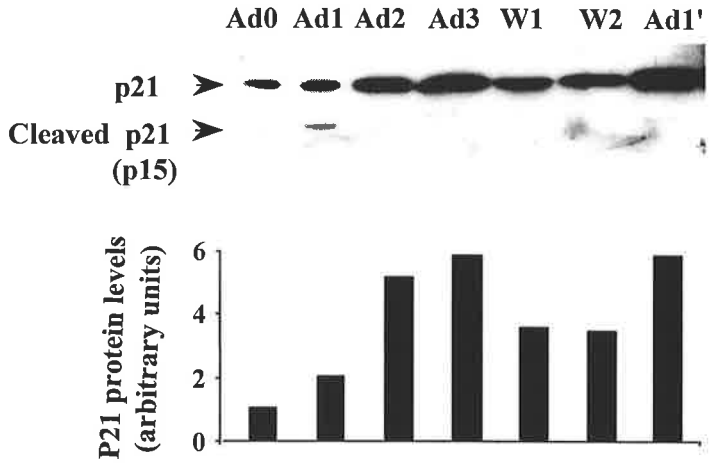


Figure 5.6 Expression of p21 and cleaved p21 in butyrate-resistant LIM 1215 cells. Western blots with anti-p21 were performed for corresponding extracts of butyrate sensitive and resistant cells as used in Fig 5.2. 36 μg protein was loaded in each lane. The figure shows an increase in p21 expression during induction of resistance by butyrate (Ad2 and Ad3) and a decrease in p21 during the withdrawal period (W1 and W2). Note also the strong signal of cleaved p21 in Ad1 but not Ad1'. The latter had a weak band that is not evident on the photomicrograph. A densitometric profile showing the relative levels of p21 protein in each track is shown at the bottom. The results are representative of a typical experiment repeated three times.

cells were still resistant to addition of butyrate (data not shown) but p21 levels had declined. By day 2 of withdrawal of butyrate (W2), cells were now sensitive to butyrate but p21 levels were no lower than at W1. This suggests a lag of at least 24 h between decline in p21 and re-acquisition of sensitivity to butyrate. When butyrate was re-added after the withdrawal period, p21 levels rose again, and there was a faint band corresponding to p15 (lane Ad1'). These cells were now sensitive to butyrate as shown in Fig 5.2. The data suggest that induction of p21 by butyrate in colorectal cancer cells correlates to some extent with induction of resistance but correlation is not complete.

5.3 DISCUSSION

It is not yet known why, in response to butyrate, some CRC cells differentiate (Young, 1994) while others die (Calabresse *et al.*, 1993; Hague *et al.*, 1995; Medina *et al.*, 1997; Hague *et al.*, 1993). The differentiation pathway is most prominent at 1 mM butyrate (Young, 1994) while apoptosis is favored at 4 mM (Medina *et al.*, 1997). However, even at 4 mM butyrate, a significant proportion of cells remained viable. A small proportion of these were killed by a second application of butyrate but no further cell loss occurred with a third cycle of butyrate. The aim of the studies described in this chapter was to explore further the mechanism by which colorectal cancer cells become resistant to butyrate.

The key observations are as follows:

1) three cycles of addition of butyrate and removal of dead cells yields a population of cells which are resistant to butyrate;

2) these cells are also resistant to a second, structurally-unrelated inhibitor of histone deacetylase TSA;

3) butyrate has to be present continuously for resistance to be maintained;

4) the population of resistant cells has a similar, but not identical, profile for the different phases of the cell cycle;

5) 1 mM butyrate, which predominantly causes a G_1 arrest (see Chapter 3), causes only a partial induction of butyrate resistance, while 4 mM butyrate, which predominantly causes a G_2+M arrest (see Chapter 3), causes a complete induction of butyrate resistance;

6) the expression of p21, which is up-regulated by butyrate (presumably as a consequence of inhibition of histone deacetylase), is very high in the resistant cells when compared to the sensitive cells;

7) p15, a caspase cleavage product of p21, is present when sensitive cells are treated with butyrate but not when resistant cells are treated with butyrate;

8) butyrate-resistant cells remain sensitive to another apoptosis-inducing agent STS.

Several possible mechanisms to account for these observations have been considered. Firstly, butyrate can be metabolised by the mitochondria of cells and therefore, resistant cells may simply metabolise butyrate rapidly and prevent its accumulation in the cell nucleus thereby preventing inhibition of histone deacetylase. I do not believe this can explain the resistance. There are three reasons for this: 1) re-addition of fresh butyrate at daily intervals did not cause the resistant cells to apoptose; 2) acquisition of butyrate-resistance required butyrate to be present continuously and therefore if butyrate was

rapidly metabolised by the resistant cells then this condition will not be met; 3) TSA, another histone deacetylase inhibitor which is not metabolised by cells, also did not induce apoptosis in resistant cells. In order to formally disprove this hypothesis, the most direct experiment would be to add ^{14}C-butyrate to resistant (Ad3) and sensitive (Ad0) cells and then measure ^{14}C-labeled metabolites. There was not sufficient time to perform this experiment.

A second possible mechanism is that histone deacetylase, the major cellular target of butyrate, is no longer inhibited by butyrate in butyrate-resistant cells. In support of this hypothesis, the cells were cross-resistant to another inhibitor of histone deacetylase TSA. If butyrate-resistant cells have some alteration in histone deacetylase that renders this enzyme resistant to butyrate then the same alteration may also prevent inhibition by TSA. Since the structures of butyrate and TSA are quite different, these agents probably inhibit histone deacetylase by different mechanisms. The sensitivity of the cells to STS, which is a protein kinase inhibitor, also fits with this hypothesis. While these observations support the hypothesis that histone deacetylase may be defective in resistant cells, it is not consistent with two other observations. Firstly, if histone deacetylase is not capable of being inhibited by butyrate in the resistant cells why is there more, rather than less, p21 expression in these cells? Secondly, why does butyrate have to be present continuously for resistance to be maintained? The implication of this is that whatever defect there is in histone deacetylase in the resistant cells, this defect is only apparent when butyrate is present. It is not possible at this stage to determine whether the resistance to butyrate is mediated at the level of histone deacetylase, or at a downstream step, but this could be

formally tested by assaying the levels and activity of this enzyme in resistant and sensitive cells before and after butyrate treatment.

A third possible mechanism is that the resistant cells are in some altered phase of the cell cycle which is no longer sensitive to butyrate. After 3-4 cycles of butyrate treatment, when the cells were butyrate-resistant, there was a small shift in the flow cytometric profile suggestive of a G_2+M arrest in resistant cells. By contrast, in cells treated with 1mM butyrate, where there was only a partial resistance, there was a G_1 arrest. However, these effects were relatively small and there does not appear to be a good correlation between butyrate-resistance and cell cycle phase. In support of this hypothesis, LIM1215 cells in G_1 arrest, induced by serum starvation, were not resistant to butyrate (data not shown).

Another possible mechanism is that the resistant cells secrete some factor which renders them resistant to butyrate. This would explain why butyrate needs to be continuously present for resistance to be maintained, if the factor is induced by butyrate. Butyrate is known to stimulate the secretion of some enzymes eg plasminogen activator inhibitor 1 (Antalis and Reeder, 1995; Gibson *et al.*, 1994; Gibson *et al.*, 1999). A way of testing this hypothesis would be to culture the sensitive cells in medium conditioned by the resistant cells and then assay for caspase activation following the addition of butyrate.

Another mechanism is that butyrate up-regulates the expression of an anti-apoptotic factor in the cytoplasm or nucleus of the cells and this prevents the activation of caspases. The increase in p21 expression in butyrate-resistant cells and its decrease in the absence of butyrate during the withdrawal phase, implicates p21 as that factor. It is known that induction of p21 by butyrate is through a mechanism involving histone

hyperacetylation (Archer *et al.*, 1998). Recently, studies have suggested that p21 protect cells from apoptosis. G_1 cell cycle arrest resulting from p21 can prevent apoptosis following UV-irradiation or treatment with an RNA polymerase II inhibitor, while serum starvation, which also synchronized cells in G_1 but did not induce p21, did not protect cells from apoptosis (Bissonnette and Hunting, 1998). Vogelstein and his colleagues (Polyak *et al.*, 1996) showed that expression of p53 in a series of colorectal cancer cell lines yielded growth arrest in some lines and apoptosis in others. Inactivation of p21 by homologous recombination converted cell growth to apoptosis, suggesting p21-mediated growth arrest can protect cells from apoptosis. p21 was shown to bind to procaspase-3, forming a complex which inhibits activation of caspase-3, resulting in resistance to apoptosis (Suzuki *et al.*, 1998). Related to this point are the known effects of p21 in multidrug resistance. Cellular levels of p21 inversely correlated with apoptotic sensitivity to cis-diammedichloroplatinum with high levels of p21 expressed in drug-resistant ME-180 cervical carcinoma cells (Donato *et al.*, 2000).

However, the correlation between the levels of p21 and resistance to butyrate is not complete. In order to test this further, sensitive cells will be transiently transfected with p21cDNA to induce over-expression of p21 and the effects on butyrate-induced apoptosis determined (see Chapter 6). The effects on other anti-apoptotic factors (eg. Bcl-2) also need to be determined. Any mechanism to explain the resistance to butyrate by up-regulation of anti-apoptotic factor must take into account the observation that the resistant cells still undergo apoptosis following the addition of the protein kinase inhibitor STS. It has been reported that activation of procaspase activity is the common step linking STS-induced apoptosis and butyrate-induced apoptosis (Medina *et al.*, 1997). Therefore, the

overcoming of resistance by STS suggests that butyrate-resistance occurs upstream of activation of caspase-3. It now needs to be determined which other apoptotic agents the butyrate resistant cells remain sensitive to.

The relationship of butyrate resistance in LIM1215 and SW480 cells to other examples of butyrate resistance needs clarifying. Tsujii and DuBois (1995) showed that rat intestinal epithelial cells over-expressing Cox-2 were resistant to butyrate-induced apoptosis. This does not necessarily imply that Cox-2 was responsible for the resistance since Bcl-2 was also indirectly up-regulated in these cells. Normal colonic epithelial cells may also be resistant to some effects of butyrate, although apoptosis was not studied (Tsujii and DuBois, 1995). It is also not clear whether primary colonic tumour cells are sensitive or resistant to butyrate. Since colonocytes and CRC cells are exposed to butyrate continuously at mM concentration, they may be rendered butyrate-resistance. If this is so, withdrawal of butyrate followed by re-application in vivo may enhance tumour cell death. This is however simply speculation without any evidence for support.

Some of the results in this chapter were published in Chai, et al. 2000.

CHAPTER 6

THE EFFECTS OF P21$^{WAF1/CIP1}$ AND P15 EXPRESSION IN COLORECTAL CANCER CELLS

6.1 INTRODUCTION

In chapter 4 and 5, it was shown that in butyrate-induced apoptosis of LIM1215 colorectal cancer cells, $p21^{Waf1/Cip1}$ (p21), a cyclin-dependent kinase inhibitor, is up-regulated and cleaved by caspase-3-like activity. This raises two questions: 1) Is loss of p21 required for LIM1215 cells to undergo apoptosis induced by butyrate? 2) Is the cleavage product p15 required for apoptosis?

Recently, a number of studies have suggested that p21 protects cells from apoptosis. Arrest of cells in G_1 phase of the cell cycle resulting from p21 expression prevented apoptosis following UV-irradiation or treatment with an RNA polymerase II inhibitor, while serum starvation, which also synchronized cells in G_1 but did not induce p21, did not protect cells from apoptosis (Bissonnette & Hunting, 1998). In ME-180 cervical carcinoma cells, selected for high or low apoptotic sensitivity to cis-diammedichloroplatinum, cellular levels of p21 inversely correlated with cis-diammedichloroplatinum responsiveness, with high levels of p21 expressed in drug-resistant cells (Donato et al, 2000). In A549 cells, p21 cooperated synergistically with bcl-2 to suppress caspase-mediated apoptosis induced by camptothecin (Zhang et al, 1999). Vogelstein's laboratory showed that expression of p53 in a series of colorectal cancer cell lines yielded growth arrest in some lines and apoptosis in others (Polyak, et al, 1996). Inactivation of p21 by homologous recombination converted growth arrest of cell to apoptosis. Other investigators reported that p21 overexpression inhibited Fas-dependent apoptosis in human hepatoma cells (Suzuki *et al.*, 1998,), as well as apoptosis mediated by several agents in human RKO colorectal cancer cells and melanoma cells (Gorospe *et al.*, 1997; Gorospe *et al.*, 1996). However, over-expression of p21 did not affect adriamycin-induced-apoptosis in p53 deficient DLD1 colorectal

carcinoma cells (Sheikh *et al.*, 1997); furthermore, p21 was required for induction of p53-dependent apoptosis in some cells (el Deiry *et al.*, 1994) and overexpression of p21 induced apoptosis in human breast carcinoma cell lines MCF-7 and T47D (Sheikh *et al.*, 1995). Therefore, the effects of p21 on apoptosis are complex and may depend on the cell type and signalling pathway used.

How does p21 act to protect some cells from apoptosis? One possible mechanism is that p21 prevents exit of cells from G_1 into both S phase and apoptosis (O'Connor, 1997). Alternatively, Suzuki et al (1998) showed that p21 binds to procaspase-3 and forms a complex, thereby inhibiting activation of caspase-3. Therefore, the mechanism(s) for the anti-apoptotic effects of p21 remain unclear.

In this chapter, the effect of p21 expression on butyrate-induced apoptosis was investigated further by transfection of p21 or p15 in LIM1215 cells and SW480 cells, mainly focusing on 1) Does over-expression of p21 prevent butyrate-induced apoptosis? and 2) Does the cleavage product p15 enhance apoptosis? Full-length p21cDNA and cDNA corresponding to the cleavage product p15 were cloned in frame with the enhanced green fluorescent protein (EGFP). The fusion expression constructs were then transiently transfected into LIM1215 or SW480 cells. The transfectants were treated with butyrate and apoptosis was assessed by DEVD-caspase activation and Annexin-V flow cytometry.

6.2 RESULTS

6.2.1 Generation of p21 and p15-EGFP fusion constructs

To test the effect of over expression of p21 and p15 on LIM1215 and SW480 cells, an expression plasmid, pEGFP-N1 (Clontech), was used. pEGFP-N1 encodes a red-shifted variant of wild-type GFP which has been optimized for brighter fluorescence and higher expression in mammalian cells. The multiple cloning site (MCS) in pEGFP-N1 is between the immediate early promoter of CMV ($P_{CMV\ IE}$) and the EGFP coding sequences (Fig 6.1B Left panel). Therefore, target genes cloned into the MCS will be expressed as fusions to the N-terminus of EGFP if they are in the same reading frame as EGFP and there no intervening stop codons. The full-length p21cDNA and cDNA corresponding to the cleavage product p15 were cloned in-frame with EGFP at the sites of *Eco*RI/*Bam*HI in pEGFP-N1. Fig 6.1 and Fig 6.2 show diagrammatically the strategy which is used for the generation of recombinant vectors. Briefly, full-length p21cDNA and cDNA corresponding to the cleavage product p15 were amplified from PCRII-p21 (a gift from Dr Helena Richardson, University of Adelaide, Adelaide, Australia) using the primers containing *Eco*RI or *Bam*HI site (see 2.2.10), which would allow their direct ligation in frame with the pEGFP plasmids. Restriction enzyme digestion of plasmid pEGFP, p21 and p15 PCR products by *Eco*RI and *Bam*HI is described in 2.2.11, and ligation of p21 or p15 insert into plasmid pEGFP is described in 2.2.13. Recombinant p21-EGFP or p15-EGFP were transformed into competent bacteria JM109 cells (see 2.2.14). Preparation of plasmids p21-EGFP or p15-EGFP is described in 2.2.15.

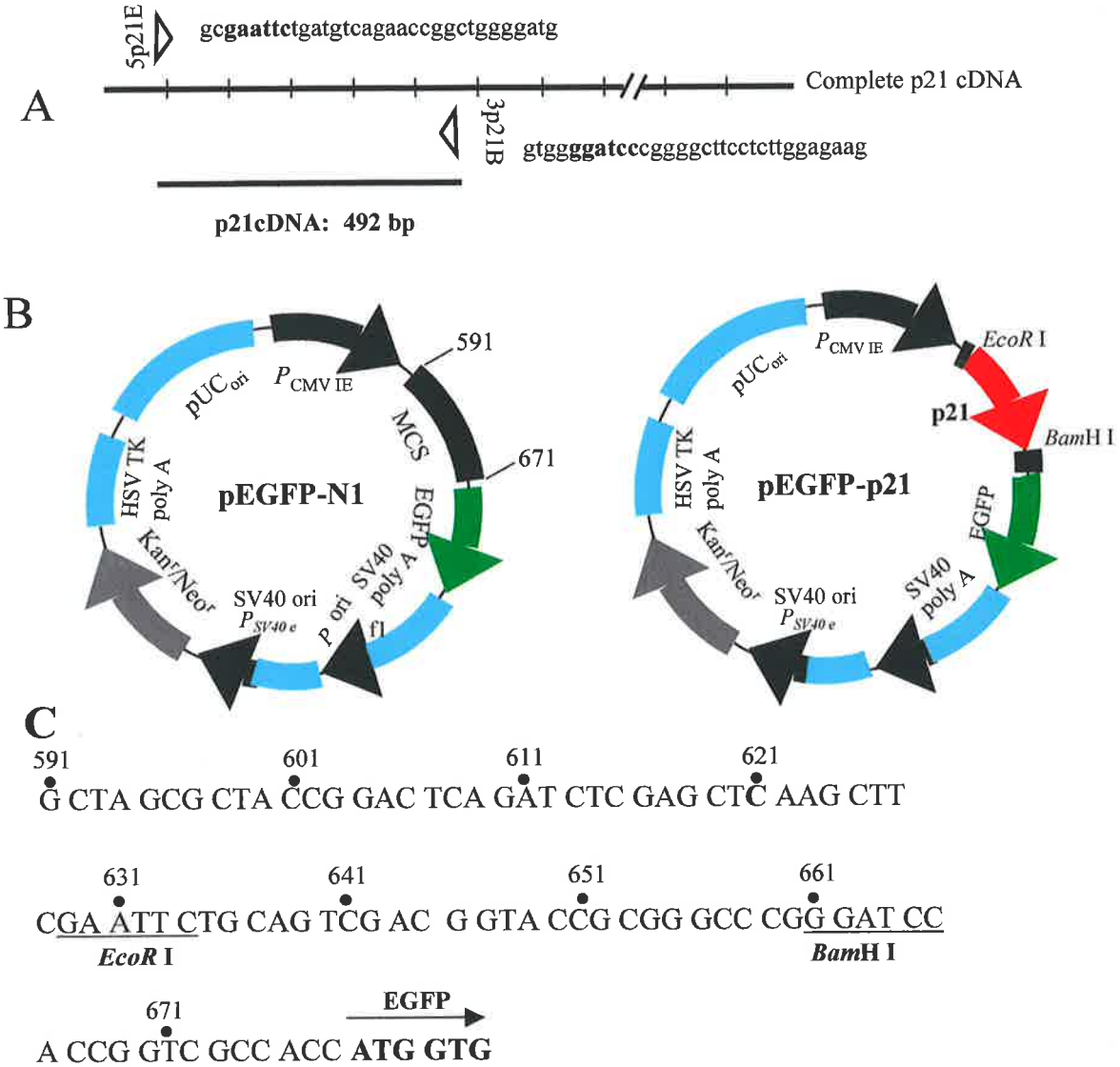


Figure 6.1 Cloning of full length p21^{Waf1}cDNA into expression vector pEGFP.

A: A full length p21 cDNA was amplified from PCRII-p21 by PCR using the primers 5p21E and 3p21B.

B: Left panel: pEGFP-N1 expression vector showing relative position of the different components. **Right panel:** p21cDNA was cloned into the MCS of the plasmid pEGFP at the sites of *EcoR* I /*Bam*HI.

C: Sequence of MCS showing the *EcoR* I /*Bam*HI restriction sites.

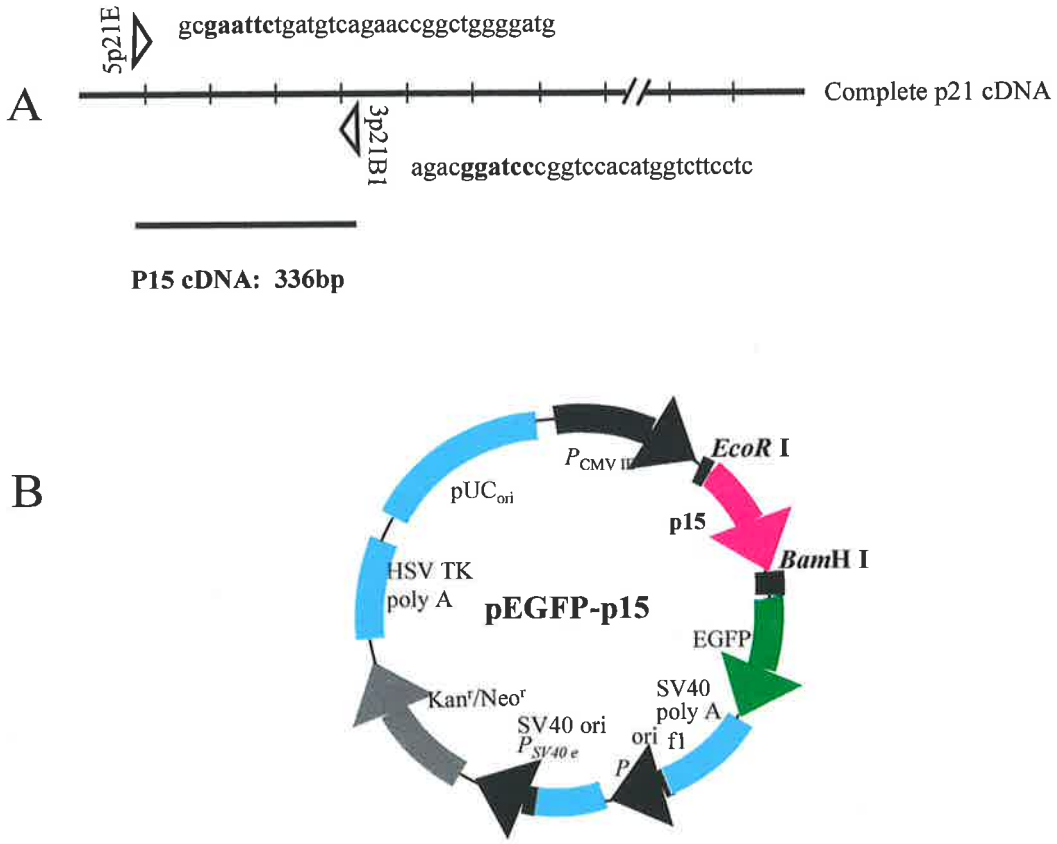


Figure 6.2 Cloning of a sequence encoding for p15 (cleavage product of $p21^{Waf1}$) into expression vector pEGFP.

A: A 336 bp fragment encoding for p15 was amplified from PCRII-p21 by PCR using the primers 5p21E and 3p21B1.

B: p15cDNA was cloned into the MCS of the plasmid pEGFP at the sites of *EcoR* I /*Bam*HI.

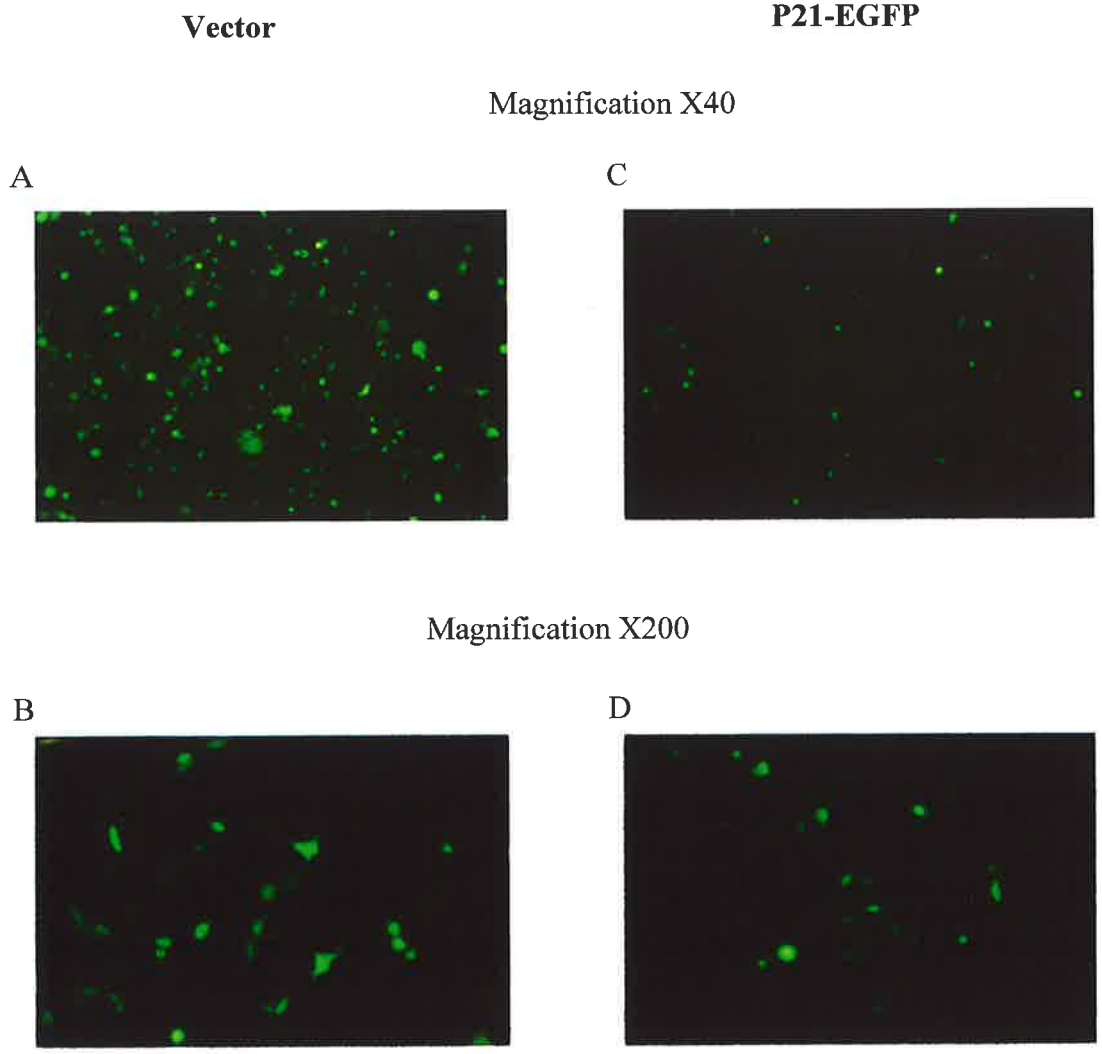


Figure 6.3 Fluorescence micrographs of LIM1215 cells transfected with pEGFP vector or pEGFP-p21. LIM 1215 cells were transfected with pEGFP vector (A&B) or pEGFP-p21(C&D) for 72 h in 6-well plates and then examined by fluorescence microscopy using the FITC filter at low magnification (40X A&C) or higher magnification (200X B&D). Typical micrographs are shown. Note the decreased number of fluorescent cells where cells were transfected with p21 consistent with growth arrest.

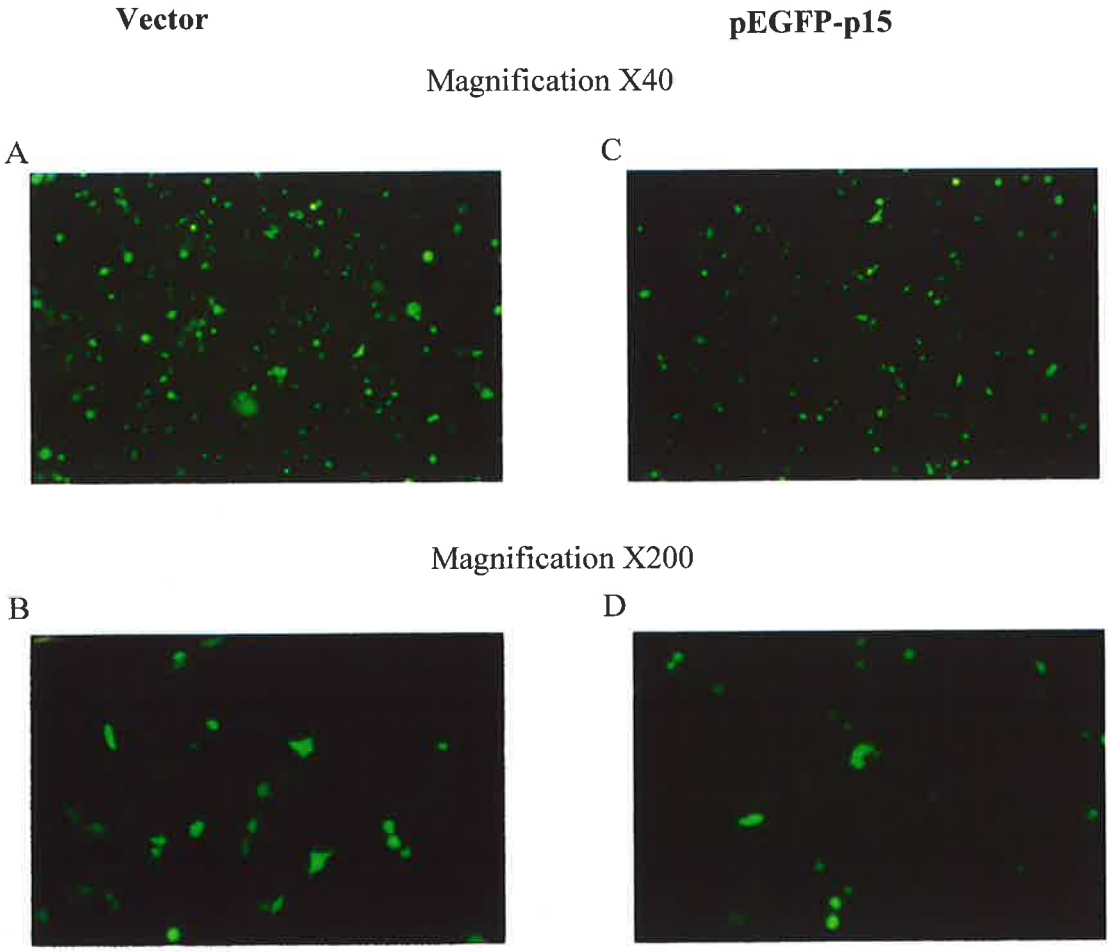


Figure 6.4 Fluorescence micrographs of LIM1215 cells transfected with pEGFP vector or pEGFP-p15. LIM 1215 cells were transfected with pEGFP vector (A&B) or pEGFP-p15 (C&D) for 72 h in 6-well plates and then examined by fluorescence microscopy using the FITC filter at low magnification (40X A&C) or higher magnification (200X B&D). Typical micrographs are shown. Note that as with p21, there was a decreased number of fluorescent cells following p15 transfection but this decrease was not as large as where cells were transfected with p21. It is not clear whether this decrease is due to growth arrest or whether p15 promotes apoptosis of the cells.

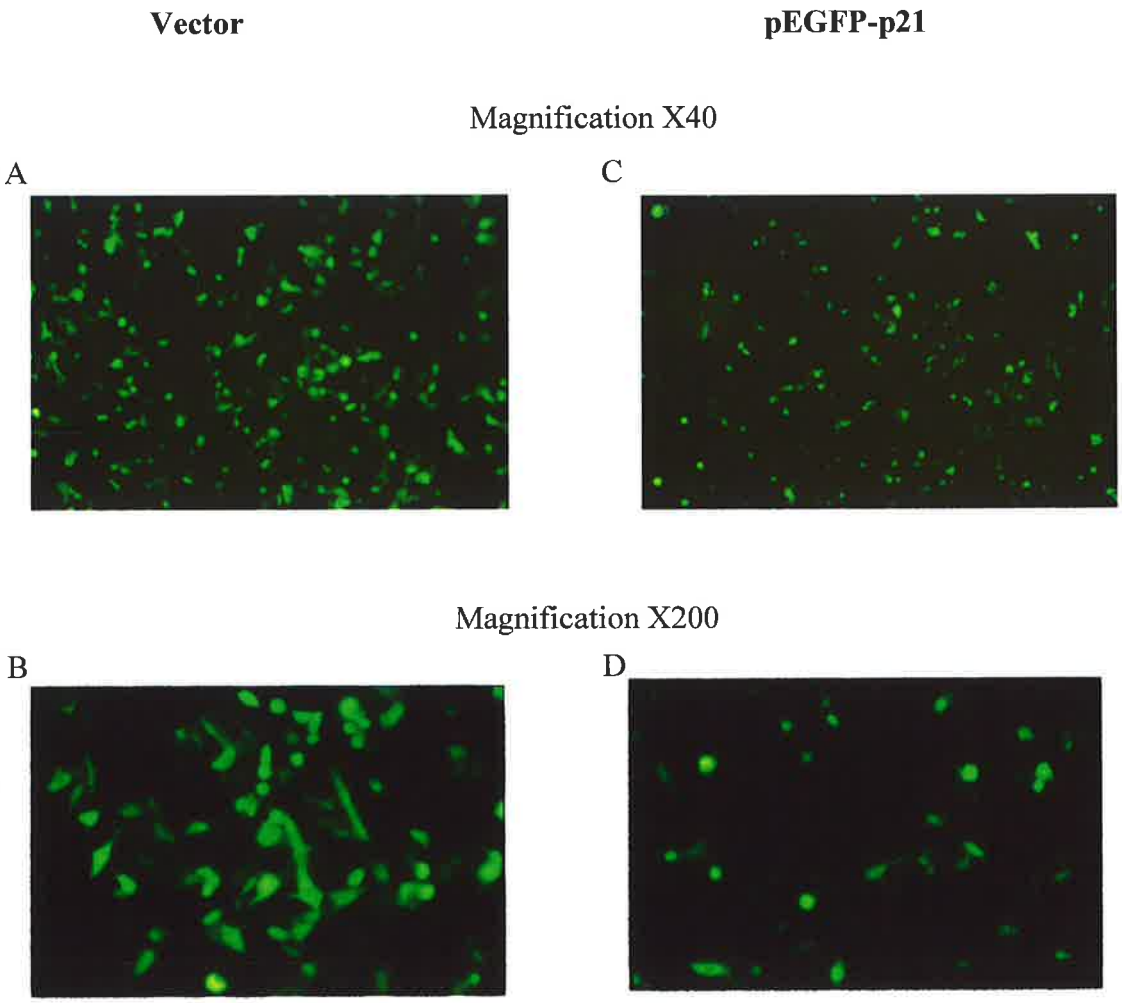


Figure 6.5 Fluorescence micrographs of SW480 cells transfected with pEGFP vector or pEGFP-p21. SW480 cells were transfected with pEGFP vector (A&B) or pEGFP-p21(C&D) for 72 h in 6-well plates and then examined by fluorescence microscopy using the FITC filter at low magnification (40X A&C) or higher magnification (200X B&D). Typical micrographs are shown. Note as for LIM1215 cells, there was a decreased number of SW480 fluorescent cells following transfection with p21 consistent with growth arrest.

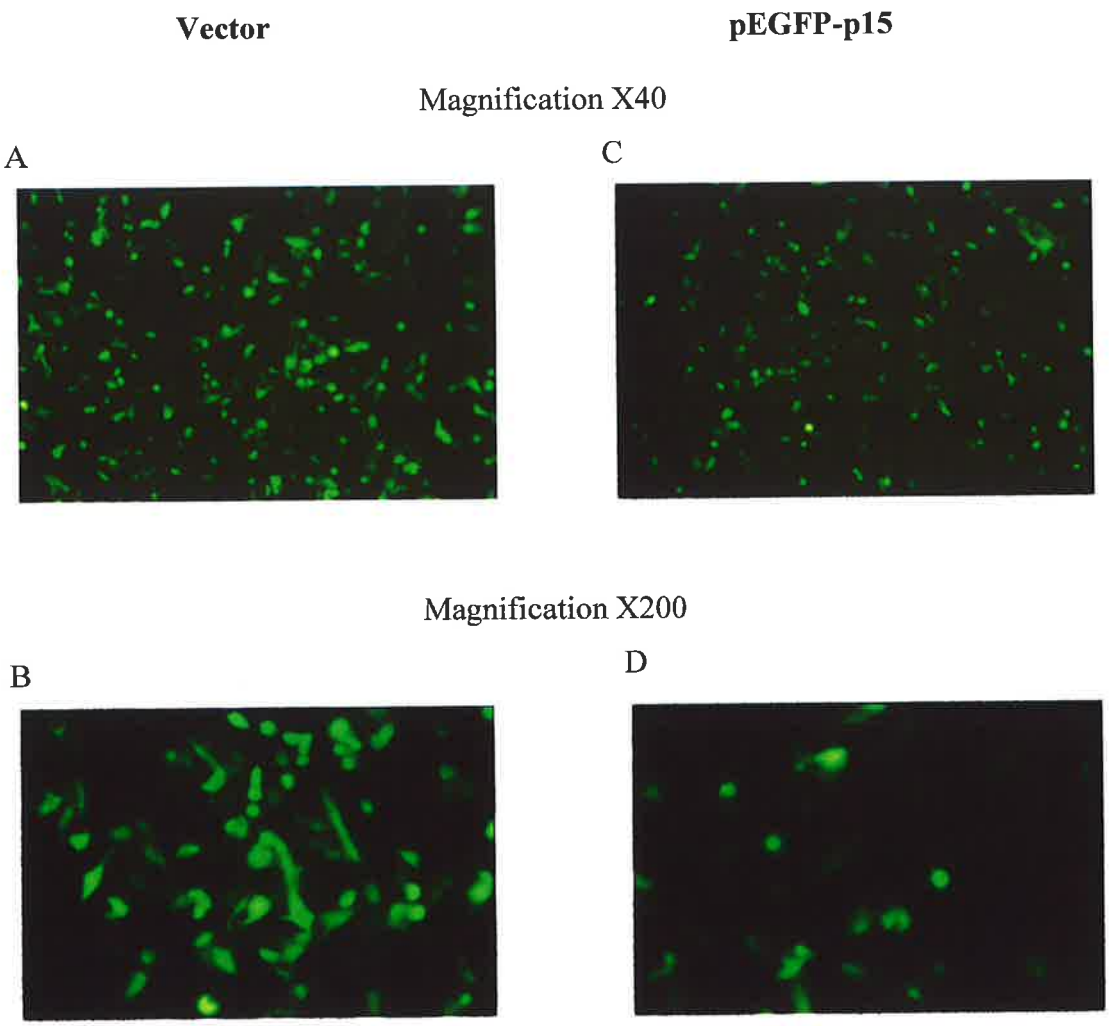


Figure 6.6 Fluorescence micrographs of SW480 cells transfected with pEGFP vector or pEGFP-p15. SW480 cells were transfected with pEGFP vector (A&B) or pEGFP-p15 (C&D) for 72 h in 6-well plates and then examined by fluorescence microscopy using the FITC filter at low magnification (40X A&C) or higher magnification (200X B&D). Typical micrographs are shown. Similar to LIM1215 cells, p15 transfection has resulted in a decreased number of fluorescent cells and this decrease was not as large as where cells were transfected with p21. Again, as in Fig 6.4, it is not clear whether this decrease is due to growth arrest or whether p15 promotes apoptosis of the cells.

6.2.2 Overexpression of p21 and p15 in LIM1215 and SW480 cells

The recombinant p21-EGFP or p15-EGFP expression plasmids were transfected into LIM1215 and SW480 cells using LIPOFECTAMINE^{TM} 2000 reagent. Green fluorescence is a marker of successful transfection. Fig 6.3 – Fig 6.6 shows fluorescence micrographs of transfected cells. Transfection efficiencies (% of total cells that express EGFP) were measured by flow cytometry as described in the legend to Table 6.1. For vector alone, p21-EGFP and p15-EGFP in LIM1215 cells, these were 12.5%, 8.6% and 9.7%, respectively. Transfection efficiencies of vector alone, p21-EGFP and p15-EGFP in SW480 cells were 24.4%, 12.3% and 15.3%, respectively.

The apparent differences in transfection efficiencies between vector and vector containing p21 or p15 may be due to the growth suppressing properties of p21 (Harper *et al.*, 1993) or p15 on these cells. As seen in Fig 6.3 and Fig 6.5, the number of fluorescent cells after 72 h of transfection was much less in the population of cells transfected with p21 compared with vector alone. It is likely that this is due to growth arrest and suggests that the p21-EGFP fusion protein still retains p21 functional activity. Similarly, the number of fluorescent cells was also less in populations transfected with p15 compared with vector alone (Fig 6.4 and Fig 6.6), although the decrease was not as much as for p21.

6.2.3 The effect of p21 and p15 transfection on butyrate-induced apoptosis.

To test the effect of over-expression of p21 or p15 on butyrate-induced apoptosis, vector alone, p21-EGFP or p15-EGFP were transiently transfected into LIM1215 and SW480 cells. Transfectants were allowed to grow for 72 h prior to treatment with 0 or 4 mM butyrate for a further 18 h. Cells were harvested and apoptosis was assessed by DEVD-caspase activity and Annexin V-PE flow cytometric analysis.

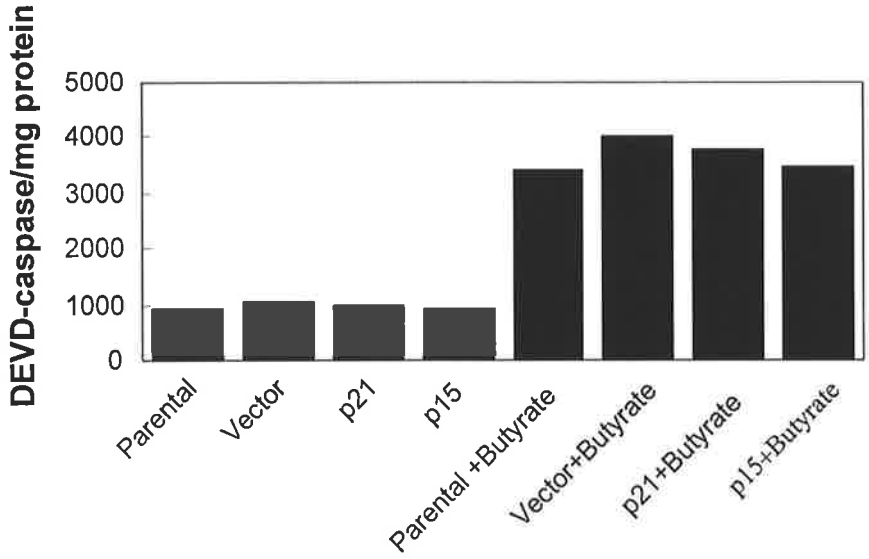


Figure 6.7 DEVD-caspase activity in p21 and p15 transfected LIM1215 cells. Cells were transfected with pEGFP vector alone, pEGFP-p21, or pEGFP-p15 for 72 h. The fluorescence of these cells is shown in Fig 6.3 and Fig 6.4. Cells were then incubated with or without 4 mM butyrate for a further 18 h. Cells were then collected and assayed for DEVD-caspase activity. This graph shows that transfection with p21 or p15 neither affects the basal levels of DEVD-caspase activity, nor the increases in DEVD-caspase activity induced by butyrate. Data represent a typical experiment repeated twice. The DEVD-caspase activity was similar for each experiment.

In the absence of butyrate, DEVD-caspase activities in parental LIM1215 cells, or cells containing vector alone, p21-EGFP or p15-EGFP were 944, 1036, 991 and 893 U /mg protein, respectively. These values are means of duplicates for different wells and represent a typical experiment. In the presence of 4 mM butyrate, DEVD-caspase activities in parental LIM1215 cells, or cells containing vector alone, p21-EGFP or p15-EGFP transfectants were 3408, 4003, 3800 and 3503 U /mg protein, respectively (Fig 6.7). Although there was a significant increase in DEVD-caspase activation in response to butyrate, there was no significant difference in caspase activation between cells expressing vector, p21 or p15.

When the cells were analysed for apoptosis by Annexin V-PE flow cytometry, even in the absence of butyrate, there appeared to be a high background of apoptotic cells (29.6%, 28.8%, 28.3% of the EGFP positive transfectants, respectively) in all three transfected populations. In butyrate-treated cells, 34.3% of the transfectants were apoptotic, that is an increase of 4.7% (Table 6.1).

What was surprising was the discrepancy between the DEVD-caspase activity and flow cytometric analysis since both assays used the same batch of cells. The reason for this discrepancy is not known but may be due to the low transfection efficiencies in LIM1215 cells. Thus, in the absence of butyrate, the cells that were Annexin V positive, GFP positive constituted only 3.7% of the total cells. In cells transfected with p21 or p15 and treated with butyrate, the percent of transfectants that were apoptotic was only slightly less than for vector alone. Time did not permit further studies to investigate this.

Because of the low transfection efficiency in LIM1215 cells, these experiments were repeated in the SW480 cell line in which relatively high transfection efficiency was

Table 6.1 The effects of transfected p21 and p15 on butyrate-induced apoptosis of LIM1215 cells

	Butyrate*	D1** Annexin-V +ve GFP -ve	D2 Annexin-V +ve GFP +ve	D3 Annexin-V -ve GFP -ve	D4 Annexin-V -ve GFP +ve	% of transfectant cells that are apoptotic
Untransfected	-	3.6	0.08	96.2	0.06	NA***
Vector alone	-	7.9	3.71	79.6	8.82	29.6
p21	-	8.4	2.49	83	6.15	28.8
p15	-	11.2	2.73	79.2	6.92	28.3
Untransfected	+	10	0.09	89.7	0.04	NA
Vector alone	+	12.4	2.99	78.9	5.73	34.3
p21	+	12.6	1.7	3.71	3.71	31.4
p15	+	12.3	2.23	80.8	4.72	32.1

* Cells treated with 4mM butyrate for 18 h

** D1-D4: LIM1215 cells were transfected with vector alone, p21 vector, or p15 vector for 3 days prior to addition of butyrate for a further 18 h. Cells were then collected and stained with Annexin-V antibody and analysed by dual labelling flow cytometry. Percentage of cells in each fraction that are + or – for green fluorescent protein (GFP) or Annexin-V are shown in the table.

*** NA: not applicable

10^5 cells were analysed. Data represent a typical experiment performed twice. The percentage of apoptotic cells was similar for each experiment.

achieved. As for LIM1215 cells, butyrate induced DEVD-caspase activity and this was not suppressed by p21 or p15 over-expression. In the absence of butyrate, DEVD-caspase activities in parental SW480 cells, or cells containing vector alone p21-EGFP or p15-EGFP transfectants were 135, 325, 269 and 301 U /mg protein, respectively (Fig 6.9). These values are means of duplicates for different wells and represent a typical experiment. In the presence of 4 mM butyrate, DEVD-caspase activities in parental SW480 cells, or cells containing vector alone, p21-EGFP or p15-EGFP transfectants were 4504, 3433, 3333 and 4075 U /mg protein, respectively (Fig 6.8).

As for LIM1215 cells, AnnexinV-PE flow cytometric analysis was also performed in SW480 cells. Unlike what was seen in the LIM1215 cells, in the absence of butyrate, there was minimal apoptosis (7%-8%) in cells transfected with vector or vector containing p21 or p15. In the presence of butyrate, apoptosis increased to 12.8% and there was no effect of over-expression of p21 or p15 on this (Table 6.2). Collectively, these data suggest that p21 and p15 do not suppress or promote butyrate-induced apoptosis in LIM1215 cells or SW480 cells. However, further studies will be needed to confirm this.

Finally, these experiments were repeated with SW480 cells that had first been sorted for EGFP positivity. SW480 cells were transfected with vector alone, p21-EGFP and p15-EGFP and allowed to grow for 72 h. Cells were then collected, sorted for EGFP positive cells by flow cytometry and reseeded to wells again for 72 h prior to addition of 0 or 4 mM butyrate. Table 6.3 shows the results of flow cytometric analysis of these cells. In absence of butyrate, percentage of apoptotic cells were 7%, 3.4%, 5.1%, and 6.6%, respectively. Following butyrate treatment, the percentage of apoptotic cells were 38%, 35%, 28% and 30%, respectively in population of untransfected cells, vector, p21-EGFP

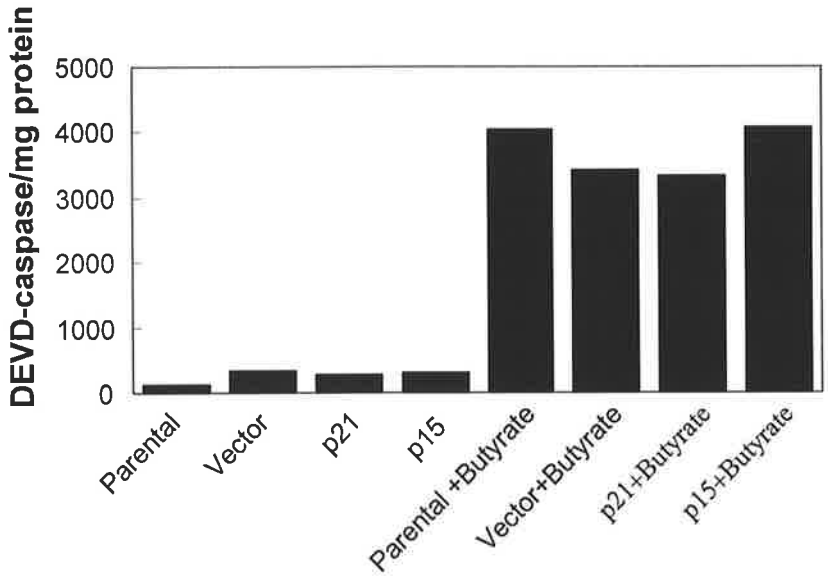


Figure 6.8 DEVD-caspase activity in p21 and p15 transfected SW480 cells. Cells were transfected with pEGFP vector alone, pEGFP-p21, or pEGFP-p15 for 72 h. The fluorescence of these cells is shown in Fig 6.5 and Fig 6.6. Cells were then incubated with or without 4 mM butyrate for a further 18 h. Cells were then collected and assayed for DEVD-caspase activity. As in Fig 6.7, this graph shows that transfection with p21 or p15 does not affect the basal levels of DEVD-caspase activity, nor the increases induced by butyrate. Data represent a typical experiment repeated twice. The DEVD-caspase activity was similar for each experiment

Table 6.2 The effects of transfected p21 and p15 on butyrate-induced apoptosis of SW480 cells

	Butyrate*	D1** Annexin-V +ve GFP -ve	D2 Annexin-V +ve GFP +ve	D3 Annexin-V -ve GFP -ve	D4 Annexin-V -ve GFP +ve	% of transfectant cells that are apoptotic
Untransfected	-	4.7	0.14	95.1	0.06	
Vector alone	-	3.6	1.74	72	22.7	7.1
p21	-	5.4	1.02	82.3	11.3	8.3
p15	-	5.6	1.03	79	14.3	6.7
Untransfected	+	21.5	0.32	77.9	0.27	
Vector alone	+	10.2	3.64	61.4	24.8	12.8
p21	+	12.8	1.94	71.6	13.7	12.4
p15	+	13.5	2.48	66.3	17.7	12.3

* Cells treated with 4 mM butyrate for 18 h

** D1-D4: . SW480 cells were transfected with vector alone, p21vector, or p15 vector for 3 days prior to addition of butyrate for a further 18 h. Cells were then collected and stained with Annexin-V antibody and analysed by dual labelling flow cytometry. Percentage of cells in each fraction that are + or – for green fluorescent protein (GFP) or Annexin-V are shown in the table.

10^5 cells were analysed. Data represent a typical experiment performed twice. The percentage of apoptotic cells was similar for each experiment.

Table 6.3 The effects of transfected p21 and p15 on butyrate-induced apoptosis of sorted GFP-positive SW480 cells

	Butyrate*	D1** Annexin-V +ve GFP -ve	D2 Annexin-V +ve GFP +ve	D3 Annexin-V -ve GFP -ve	D4 Annexin-V -ve GFP +ve	% apoptotic cells***
Untransfected	-	6.91	0.09	92.95	0.05	7.0
Vector alone	-	2.17	1.2	66.75	29.9	3.4
p21	-	4.93	0.18	93.95	0.95	5.1
p15	-	6.25	0.4	91.95	1.41	6.6
Untransfected	+	37.9	0.1	61.95	0.03	38.0
Vector alone	+	26.75	8.22	44.1	20.85	35.0
p21	+	27.95	0.43	71	0.61	28.4
p15	+	29.1	0.83	68.95	1.18	29.9

* Cells treated with 4mM butyrate for 18 h

** D1-D4: Methodology varies from that in table 6.1 and 6.2. SW480 cells were transfected with vector alone, p21vector, or p15 vector for 3 days. Then cells were collected and sorted into GFP positive and negative cells by FACS. EGFP positive cells were seeded to the wells and allowed to grow for two days prior to addition of butyrate for a further 18 h. Cells were then collected and stained with Annexin-V antibody and analysed by dual labelling flow cytometry. Untransfected cells that had not been sorted were used as controls. Percentage of cells in each fraction that are + or – for EGFP or Annexin-V are shown in the table.

*** Percent of total cells that were apoptotic cells. This was determined by the ratio of D1+D2 / D1+D2+D3+D4. This was used because theoretically all of the sorted cells were EGFP positive to start with. Note however, after culture a substantial proportion of the cells were in the D3 fraction (that is they were now EGFP negative). See discussion for further comments.

10^5 cells were analysed. Data represent a typical experiment performed twice. The percentage of apoptotic cells was similar for each experiment.

and p15-EGFP transfectants. It would appear that p21 and p15 did not significantly affect butyrate-induced apoptosis in transfectants. However, the flow cytometric analysis data showed that EGFP positive cells in p21 or p15 transfectants were very low (sum of D2+D4 cells were 1.04% and 2.01% respectively) compared with vector alone (29.07%). This may be because transfectants over-expressing p21 or p15 are growth arrested, and therefore at a disadvantage compared to cells not over-expressing p21 or p15. This would be more important for the sorting experiment since these cells are cultured for 6 days, whereas for experiments involving unsorted cells, the culture was only for 3 days. In view of the low proportion of the cells expressing p21 or p15 it is not possible to make any conclusions about their effects on butyrate induced apoptosis.

Fig 6.9 shows the DEVD-caspase activity results for the sorting experiment. The graph shows an apparent decrease in DEVD-caspase activity induced by butyrate in cells transfected with p21 or p15 relative to cells transfected with vector alone. However, the cell yields from the FACS were low and it was only possible to do duplicate tubes. Time did not permit the experiment to be repeated. Therefore, the significance of the data is unclear.

6.3 DISCUSSION

The conflicting effects of p21 on apoptosis in different cellular models has been discussed in the Introduction. In the studies in Chapter 5, p21 was upregulated in butyrate-resistant LIM1215 cells, suggesting that it may suppress apoptosis by butyrate.

Experiments in this chapter were performed to investigate the effect of p21 on butyrate-induced apoptosis of CRC cell lines. Expression vector p21-EGFP was constructed and transfected successfully into LIM1215 and SW480 cells; although the transfection efficiencies in these two cell lines were low, these were higher in SW480 cells than in

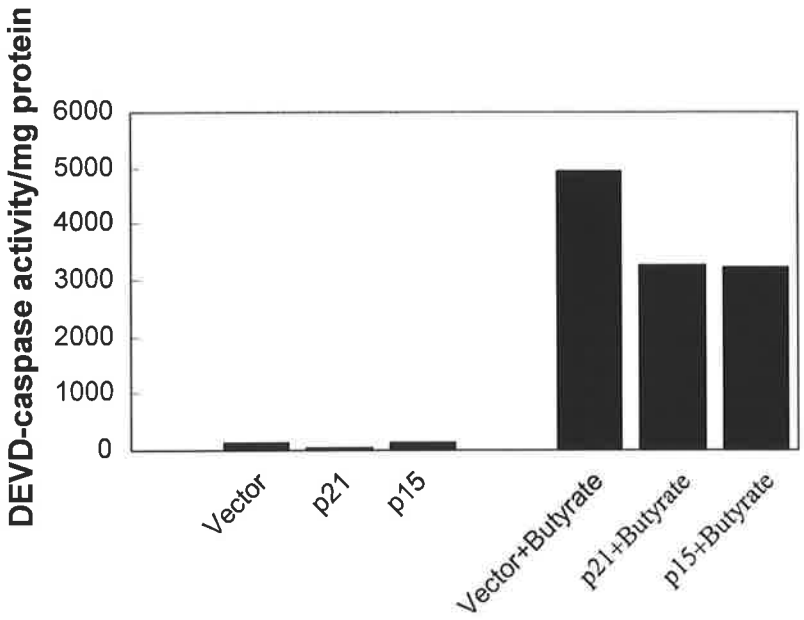


Figure 6.9 DEVD-caspase activity in sorted p21 and p15 transfected SW480 cells. Cells were transfected with vector alone, pEGFP-p21, or pEGFP-p15 for 72 h. Then cells were collected and sorted into EGFP positive and negative cells by FACS. EGFP positive cells were seeded in wells and allowed to grow for 48 h prior to addition of 4mM butyrate for a further 18 h. Controls were incubated without butyrate. Cells were then collected and assayed for DEVD-caspase activity. The graph shows an apparent decrease in DEVD-caspase activity induced by butyrate in cells transfected with p21 or p15 relative to cells transfected with vector alone.

LIM1215 cells. However, in both LIM1215 and SW480 cells, transient over-expression of p21 did not protect against butyrate-induced apoptosis of these cells, as judged by DEVD-caspase activity and flow cytometric analysis. However, these experiments would be better repeated using an inducible expression system for p21 or p15, that would allow stable transfection without affecting the growth rate of the cells. Another approach is to use a retroviral expression system in which very high transfection efficiency can be achieved. Another potential problem with the experiment design is that p21 and p15 may not be expressed at high enough levels to suppress the apoptosis. Future studies should monitor the expression of p21 and p15 by Western blotting and immunocytochemistry. Finally, it needs to be determined whether p21 and p15 are functional in the transfectants particularly since these proteins will exist as fusion products with EGFP. The best way to test this would be by analysing the cell cycle of transfectants using flow cytometry. In cells over-expressing p21 (and possibly p15) there should be an increase in G_0/G_1 and or G_2+M cells at the expense of S cells. However, the decrease in EGFP fluorescent cells as seen by fluorescence microscopy and flow cytometry (Table 6.1-6.3) suggested both p21 and p15 were growth arresting the cells and/or promoting cell death.

In conclusion, the experiments described in this chapter do not support the hypothesis that p21 suppresses butyrate-induced apoptosis although it did appear to arrest the growth of the cells. Similarly, the transfection experiments do not support the hypothesis that p15 promotes apoptosis. However, the experiments are not ideal and the negative results may be misleading. These conclusions need to be confirmed by using other criteria for assessment of apoptosis and by using an inducible system or a retroviral expression system for over-expression of p21 and p15.

CHAPTER 7

INTERACTIONS BETWEEN BUTYRATE AND NSAIDS ON APOPTOSIS OF COLORECTAL CANCER CELL LINES

7.1 INTRODUCTION

High dietary fibre has been accepted as a preventative factor for colorectal tumours. Butyrate, a colonic fermentation product of dietary fibre, is thought to be a major contributor of this protection. Several mechanisms have been proposed for the action of butyrate. Of particular interest are its effects on stimulating apoptosis of colon cancer cell lines.

Of therapeutic importance are the following two questions. Firstly, will combinations of butyrate with other cancer chemopreventive agents have synergistic or additive effects? In order to obtain most benefits from cancer chemoprevention, combinations of two or more chemopreventive agents may be the best strategy. Secondly, why do some colon cancer lines become resistant to butyrate? These issues were addressed here by studying effects of the nonsteroidal anti-inflammatory drugs (NSAIDs) sulindac sulfide, sulindac sulfone and lipoxygenase (Lox) inhibitor nordihydroguaiaretic acid (NDGA), alone or in combination with butyrate, on apoptosis of butyrate-sensitive and- resistant populations of LIM 1215 cells. Some experiments were repeated in SW480 cells.

NSAIDs are effective chemopreventive agents in colorectal cancer. They inhibit the growth of both transplanted tumours in mice (Hial *et al.*, 1976) and those induced by chemicals and radiation (Reddy *et al.*, 1987). In addition, the NSAID sulindac has been shown to cause regression of adenomatous colorectal polyps in Gardner's syndrome (Waddell and Loughry, 1983) and in FAP patients (Winde *et al.*, 1995). The mechanism by which NSAIDs act remains unclear. NSAIDs have been found to inhibit cell

proliferation and cause cell cycle arrest (Adolphe *et al.*, 1972), as well as inducing apoptosis in cultured cell lines (Piazza *et al.*, 1995; Shiff *et al.*, 1995; Lu *et al.*, 1995; Tsujii and DuBois, 1995).

It has been proposed that NSAIDs act by inhibition of cyclo-oxygenases (Cox), resulting in decrease of prostaglandin production (Marnett, 1992). Several pieces of evidence implicate Cox-2, which is inducible by a variety of cytokines and mitogens, in colorectal tumorigenesis. Firstly, expression of Cox-2 is elevated in colorectal tumors (Sano *et al.*, 1995); secondly, a null mutation in Cox-2 dramatically reduced the number and size of the intestinal polyps in an APC knockout mouse model (Oshima *et al.*, 1996); thirdly, rat intestinal epithelial cells, which over-express Cox-2, were resistant to butyrate-induced apoptosis (Tsujii and DuBois, 1995).

The effects of Cox-2 on tumour formation may be mediated by enhanced prostaglandin formation. However, it has also been shown that NSAIDs inhibit proliferation and induce apoptosis in colon cancer cells that lack cyclo-oxygenase transcripts and do not produce prostaglandins even when exogenously stimulated (Hanif *et al.*, 1996). Furthermore, sulindac sulfone only weakly inhibits Cox; however, it still has anti-neoplastic properties (Piazza *et al.*, 1995). Additional evidence suggesting that NSAIDs do not act via the effects on prostaglandin synthesis are that prostaglandins cannot rescue NSAID-induced growth arrest and apoptosis (Hanif *et al.*, 1996; Narisawa *et al.*, 1984; Chan *et al.*, 1998) and, in transformed embryonic fibroblast cells with knockouts of both Cox-1 and Cox-2, NSAIDs are still anti-proliferative as well as capable of inducing apoptosis (Zhang *et al.*, 1999).

Alternatively, NSAIDs may act by increasing the prostaglandin precursor arachidonic acid (AA). Treatment of colorectal cancer cells with NSAIDs has been shown to result in a dramatic increase in AA, that in turn stimulated the conversion of sphingomyelin to ceramide, a known mediator of apoptosis (Chan *et al.*, 1998). AA is usually metabolized to either prostanoids (thromboxanes and prostaglandins) via the enzyme Cox, or to leukotrienes via the enzyme Lox.

These metabolites may also play a role in the mechanism by which colon cancer cells become resistant to butyrate. In addition to the involvement of p21 in this resistance, Cox-2 and/or Lox may play a role. Possible mechanisms of butyrate resistance include up-regulation of Cox-2 (to increase PGE_2) and Lox (to increase HETEs). Evidence that Lox may be important includes the following: Lox is an essential regulator of cell survival and apoptosis in rat Walker W256 carcinosarcoma cells (Tang *et al.*, 1996), while butyrate is an inducer of Lox in colorectal carcinoma Caco-2 cells (Kamitani *et al.*, 1998).

There were two major aims of this study. The first was to further characterise the mechanisms mediating butyrate-induced apoptosis and resistance to butyrate. The second aim was to determine the interaction between butyrate and NSAIDs on apoptosis in CRC cells. Two derivatives of sulindac were used: sulindac sulfide, which inhibits Cox-1 and -2, and sulindac sulfone, which has little effect on Cox enzymes. As Lox inhibitor, NDGA was used.

7.2 RESULTS

7.2.1 Sulindac sulfide and sulfone induced apoptosis in concentration- and time-dependent manner

First, I determined the effects of sulindac sulfide and sulfone on apoptosis of colorectal cancer cell line LIM1215, using DEVD-caspase activity and DNA fragmentation as markers of apoptosis. In LIM1215 cells, induction of caspase-3 activity and DNA fragmentation by sulindac sulfide was slightly increased at 100-150 μM and was maximal at 200 μM. Treatment with 200 μM sulfide for 24 h induced a 4-5 fold increase in DEVD-caspase and DNA fragmentation. The concentration curve plateaued at >200 μM (Fig 7.1A). For sulindac sulfone, induction of DEVD-caspase activity and DNA fragmentation was slightly increased at 300-400 μM and maximal at >800 μM. Incubation with 800 μM sulfone for 24 h resulted in about 3-4 fold increase in DEVD-caspase activity and DNA fragmentation (Fig 7.1B). Therefore, sulindac sulfide and sulfone induced apoptosis in a concentration-dependent manner. Induction of DEVD-caspase activity by both sulindac sulfide and sulfone was consistent with that of DNA fragmentation in LIM1215 cells. Similar results were found in another colorectal cancer cell line SW480 (Fig 7.2A and Fig 7.2B). As for butyrate-treated cells (chapter 3), sulindac sulfide and sulfone induced much less DEVD-caspase activity and DNA fragmentation in this cell line.

The kinetics of increases in DEVD-caspase activity and DNA fragmentation were studied using 200 μM sulfide and 600 μM sulfone in LIM1215 cells. DEVD-caspase activity and DNA fragmentation increased quickly during the first 8 h after addition of

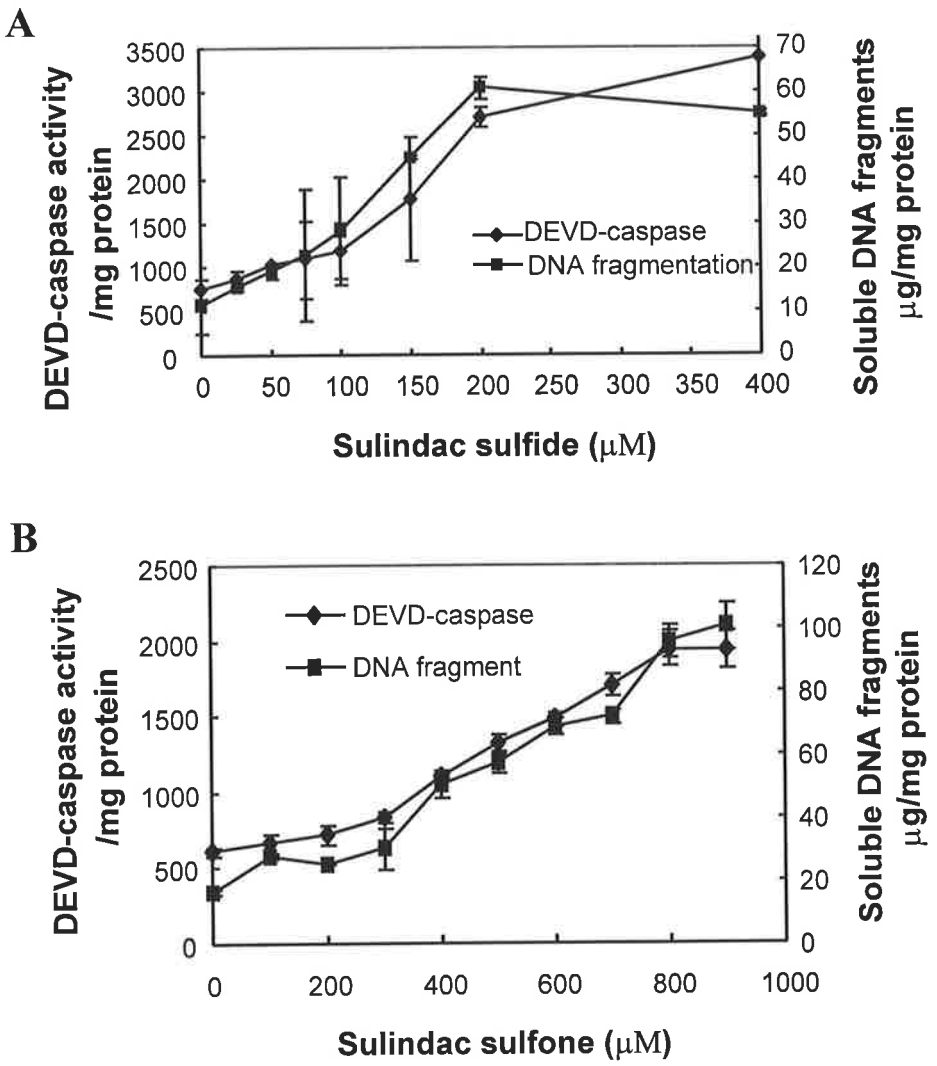


Figure 7.1 DEVD-caspase activity and DNA fragmentation induced by sulindac sulfide (A) or sulfone (B) in LIM 1215 cells. 10^6 LIM1215 cells were seeded in 25 cm^2 flask. 2-3 days after, cells were treated with different concentrations of sulindac sulfide for 24 h and then harvested for the assays of DEVD-caspase and DNA fragmentation. The graph shows that 100-150 μM sulfide began to induce DEVD-caspase activity and DNA fragmentation. Maximal induction of apoptosis was at 200 μM. With sulfone, induction of DEVD-caspase activity and DNA fragmentation began at 300-400 μM, while maximal induction occurred with more than 800-900 μM sulfone. Induction of DEVD-caspase paralleled that of DNA fragmentation. Data are means of triplicates; error bars indicate the standard deviations.

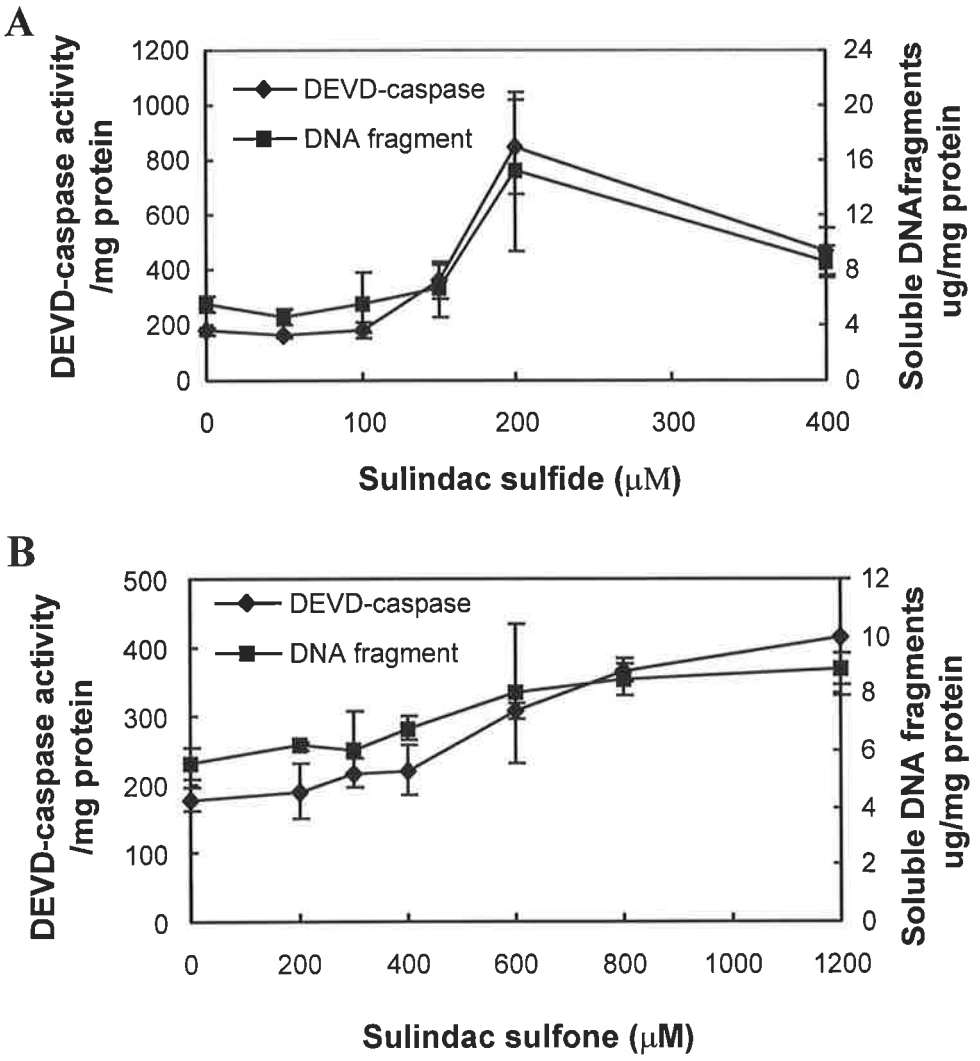


Figure 7.2 DEVD-caspase activity and DNA fragmentation induced by sulindac sulfide (A) or sulfone (B) in SW480 cells. $5x10^5$ cells were seeded into 6-well plates. Two days after, cells were incubated with sulindac sulfide for 24 h and then collected for lysis. Same lysate was used for both DEVD-caspase activity and DNA fragmentation assays. Induction of DEVD-caspase activity and DNA fragmentation began at 100-150 μM of sulfide, with maximal induction at 200 μM. Thereafter, DEVD-caspase and DNA fragmentation decreased, possibly because of secondary necrosis of sulfide treated cells. With sulfone, induction of DEVD-caspase activity and DNA fragmentation began at 300-400 μM, with maximal induction at 800 μM or greater. Data are means of triplicates; error bars indicate the standard deviations.

sulfide and reached a maximum at 24 h. After 24 h, the DEVD-caspase activity curve plateaued until 72 h and then decreased, possibly because of secondary necrosis (Fig 7.3A). The DNA fragmentation curve (Fig 7.3B) had a similar shape, but there was no decrease after 72 h. Compared to that of sulfide, induction of DEVD-caspase activity and DNA fragmentation by sulfone was slower (Fig 7.4A and Fig 7.4B). Maximal inductions were at 48 h.

7.2.2 Relationship between growth inhibition and apoptosis in colorectal cancer cells treated with sulindac sulfide and sulfone

To investigate the relationship between activation of DEVD-caspase and growth inhibition, DEVD-caspase activity was measured and compared with the total cell number (viable and non-viable) in each flask. Total cell number was inversely proportional to the induction of DEVD-caspase activity by sulfide and sulfone, respectively (Fig 7.5A and Fig 7.5B). This suggested that growth inhibition of LIM1215 cells by sulindac sulfide and sulfone was mainly due to apoptosis.

7.2.3 Caspase inhibitors block sulindac sulfide- and sulfone-induced DEVD-caspase activation but not DNA fragmentation

To determine whether caspases are involved in the apoptosis of sulindac sulfide and sulfone-treated LIM1215 cells, two specific membrane-permeable peptide caspase inhibitors were used. zDEVD-fmk is a specific inhibitor of DEVD-caspases, while zVAD-fmk is an inhibitor of a broad spectrum of caspases. Both inhibitors completely inhibited DEVD-caspase activity in sulindac sulfide and sulfone-treated LIM1215 cells (Fig 7.6), although it was not determined whether the peptide inhibitors were blocking the active

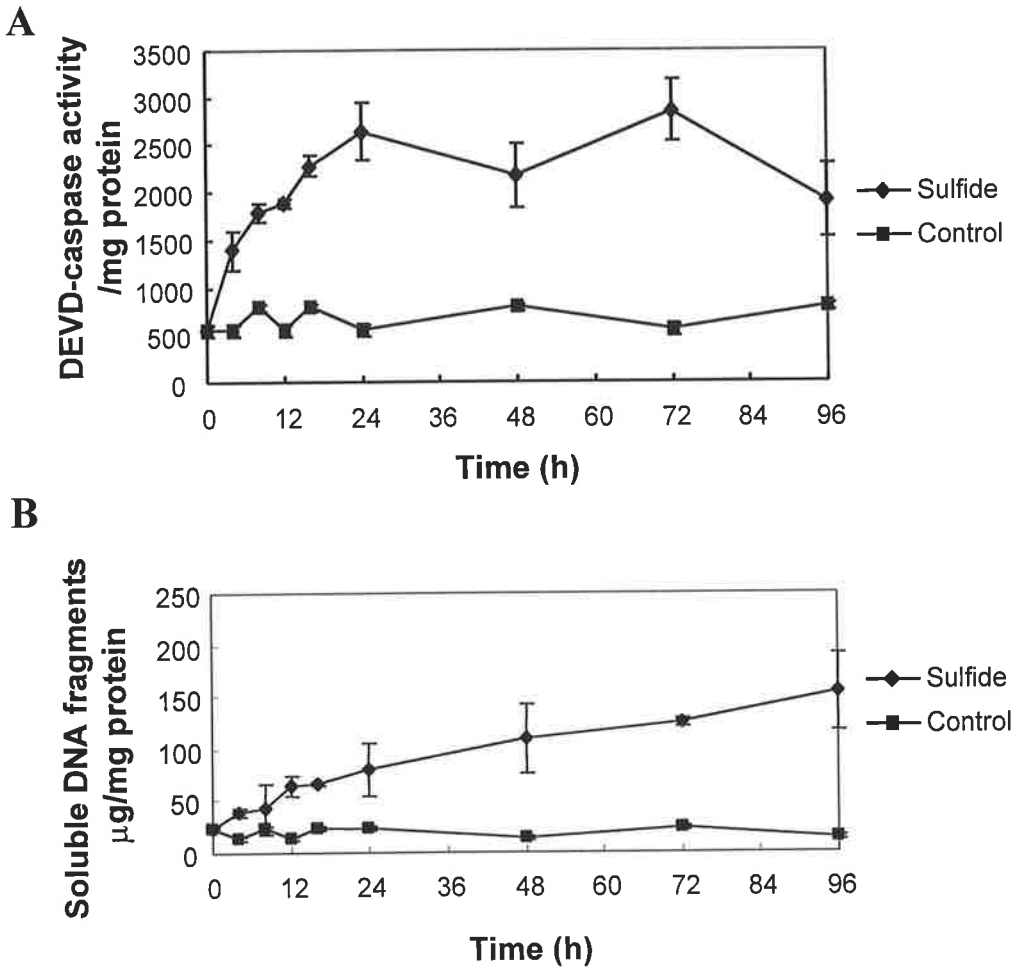


Figure 7.3 Time course of induction of DEVD-caspase (A) or DNA fragmentation (B) by 200 µM sulindac sulfide in LIM1215 cells. 1.5 x 10^6 cells were seeded in 25 cm^2 flasks. 2 days after, the cells were given fresh medium and 200 µM sulfide. At different time-points, cells were collected for DEVD-caspase activity or DNA fragmentation. This graph shows that DEVD-caspase was induced quickly within first 4 h after addition of sulfide. Maximal induction was at 24 h and maintained at high levels until 72 h. Similar results were obtained for DNA fragmentation except that maximal induction was at 48 h. Data are means of triplicates; error bars indicate the standard deviations.

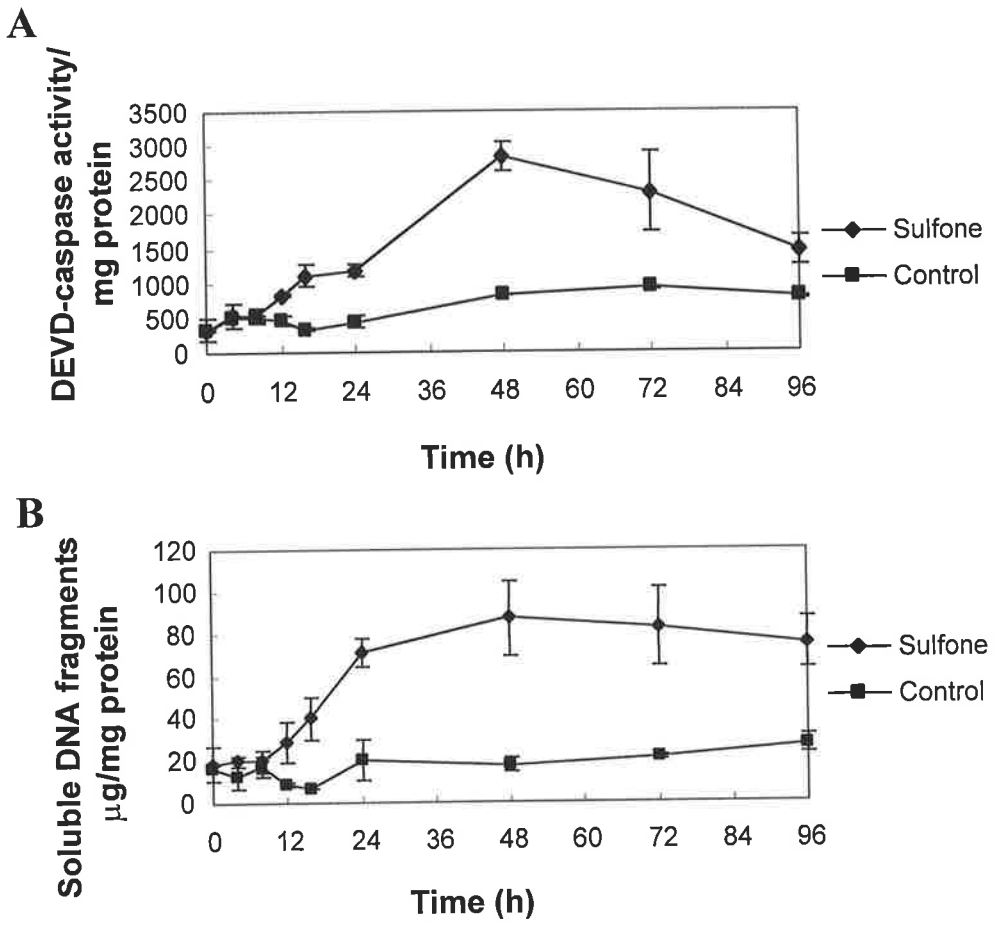


Figure 7.4 Time course of DEVD-caspase activity (A) and DNA fragmentation (B) induced by 600 µM sulindac sulfone in LIM1215 cells. 1.5 x10^6 cells were seeded in 25 cm^2 flasks (in triplicate). 2 days after, the cells were given fresh medium and 600 µM sulfone. At different timepoints, the treated cells were collected for DEVD-caspase assay and DNA fragmentation. This graph shows that induction of DEVD-caspase began at 8-12 h and then gradually increased until 48 h. Similar results were found for DNA fragmentation. Data are the means of triplicates; error bars indicate the standard deviations.

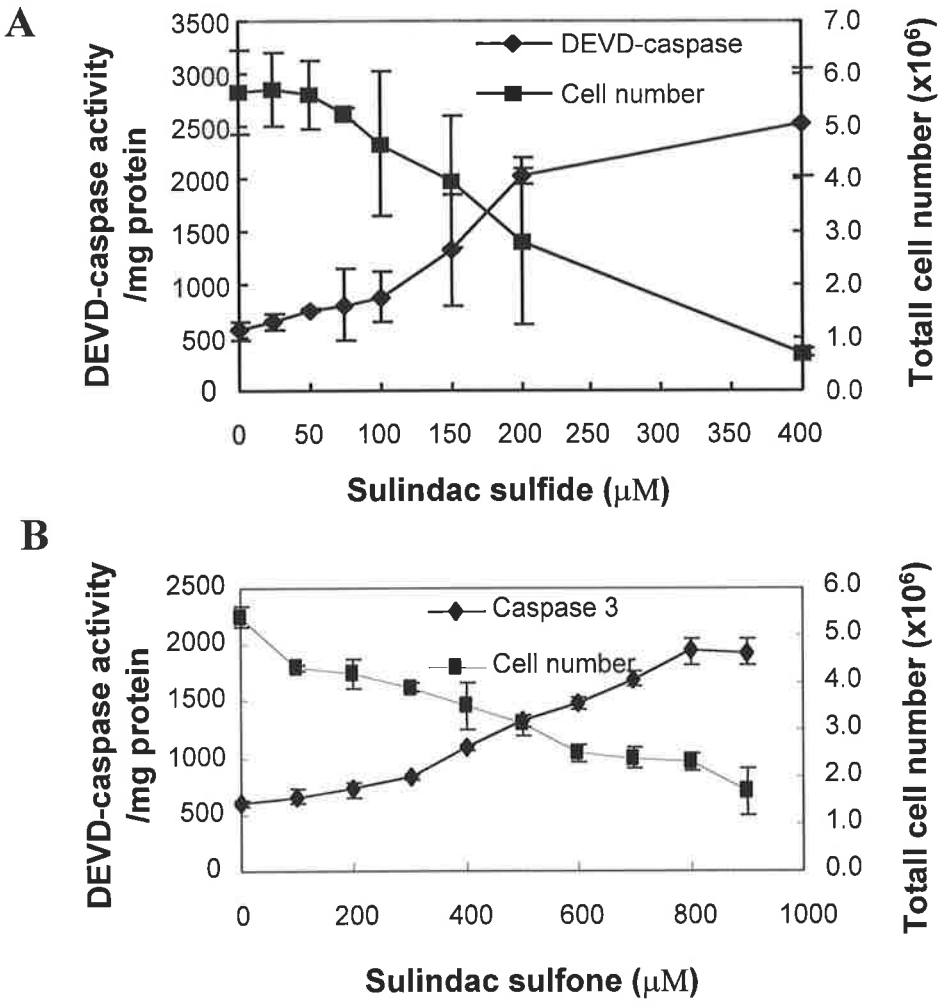


Figure 7.5 Induction of DEVD-caspase activity and growth inhibition by sulindac sulfide (A) or sulfone (B) in LIM 1215 cells. 10^6 LIM1215 cells were seeded in 25cm^2 flasks. 2 days after, cells were treated with different concentrations of sulindac sulfide for 24 h. Floating cells were collected and the remaining cells were harvested by trypsinisation and recombined with the floating cells. Total cell number was counted and then cells were lysed for DEVD-caspase activity. This figure shows that growth inhibition by sulfide or sulfone is proportional to the induction of DEVD-caspase. Data are the means of triplicates; error bars indicate the standard deviations.

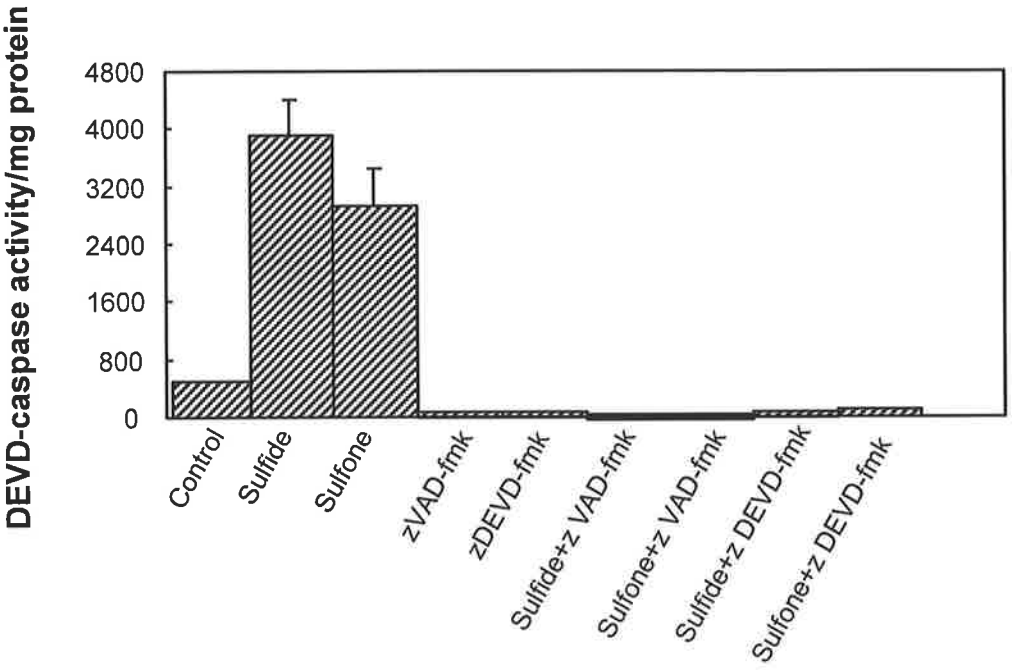


Figure 7.6 Effect of caspase peptide inhibitors on DEVD-caspase activity induced by sulindac sulfide or sulfone. 6 x10^5 LIM1215 cells were seeded into 6-well plates. Two days after, cells were pre-treated with caspase peptide inhibitors 10 μM zDEVD-fmk or 25 μM zVAD-fmk for 1 h and further incubated with 200 μM sulfide or 600 μM sulfone for 21 h. These were then harvested for DEVD-caspase activity. This graph shows that zDEVD-fmk and zVAD-fmk completely inhibit the DEVD-caspase activity induced by sulfide or sulfone. Data are the means of triplicates; error bars indicate the standard deviations.

caspase-3 or rather the caspase-dependent steps leading to activation of caspase-3. Despite the complete inhibition of DEVD-caspase activity, there was no inhibition of DNA fragmentation (Fig 7.7).

7.2.4 A combination of butyrate and sulindac sulfide or sulfone (suboptimal concentrations) resulted in synergistic effects on apoptosis.

In order to determine if there are additive or synergistic effects of sulindac derivatives and butyrate on apoptosis of colorectal cancer cells, sub-optimal and optimal concentrations of butyrate and sulindac sulfide or sulfone were used to treat LIM1215 cells for 21 h. Butyrate (1 mM) was synergistic with sulindac sulfide over a range of concentrations (Fig 7.8A). For example, a combination of 1 mM butyrate plus 50 μM sulfide yielded an increase in DEVD-caspase activity (over control) that was 126% greater than the sum of the increases in DEVD-caspase activities (over control) that were yielded by 1 mM butyrate and 50 μM sulfide individually ($p \leq 0.005$); a combination of 1 mM butyrate plus 100 μM sulfide yielded an increase in DEVD-caspase activity (over control) that was 182% greater than the sum of the increases in DEVD-caspase activities (over control) that were yielded by 1 mM butyrate and 100 μM sulfide alone ($p \leq 0.05$); a combination of 1 mM butyrate plus 200 μM sulfide yielded an increase in DEVD-caspase activity (over control) that was 67% greater than the sum of the increases in DEVD-caspase activities (over control) that were yielded by 1 mM butyrate and 200 μM sulfide alone ($p \leq 0.005$). The latter increase (67%) was smaller than the others probably because 200 μM sulfide is an optimal concentration. When an optimal concentration (4 mM) of butyrate was used, synergy was only weak or non-existent (Fig 7.8B). Thus, a combination of 4 mM butyrate and 50, or 100 μM sulfide resulted in increases of DEVD-caspase

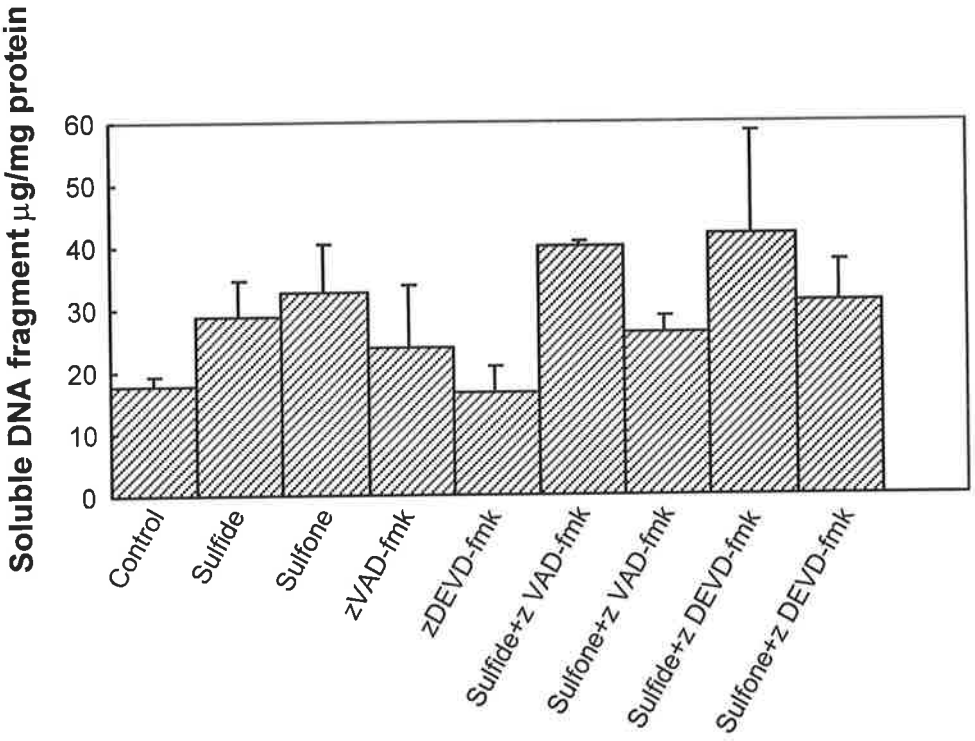


Figure 7.7 Peptide inhibitors did not block the DNA fragmentation induced by sulindac sulfide or sulfone. 6 x 10^5 LIM1215 cells were seeded into 6-well plates. Two days after, cells were pre-treated with 10 μM zDEVD-fmk or 25 μM zVAD-fmk for 1 h and further incubated with 200 μM sulfide or 600 μM sulfone for 21 h and then were harvested for DNA fragmentation. This graph shows that zDEVD-fmk and zVAD-fmk do not inhibit the DNA fragmentation induced by sulfide or sulfone. Data are the means of triplicates; error bars indicate the standard deviations.

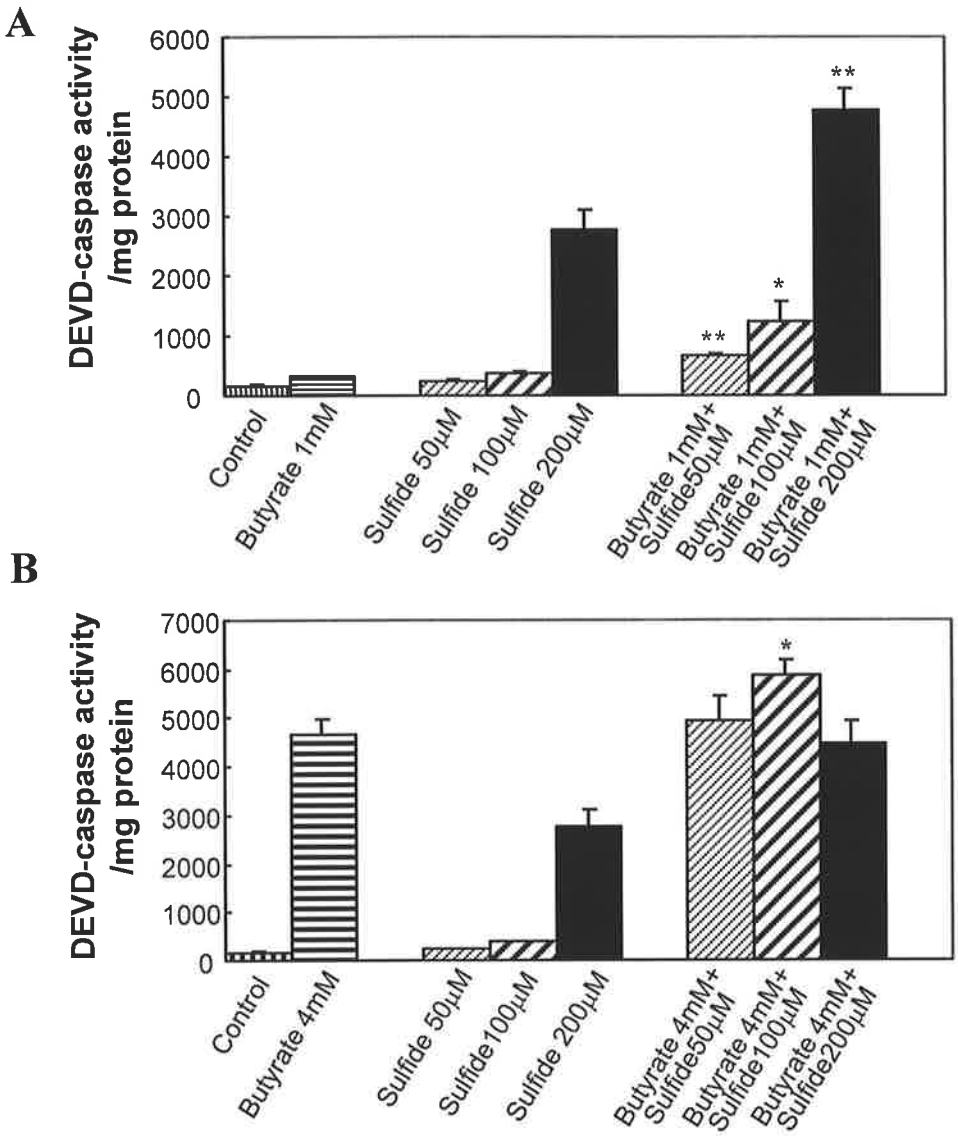


Figure 7.8 Synergistic effects of a combination of 1 mM (A) or 4 mM (B) butyrate and sulindac sulfide on DEVD-caspase activation in LIM1215 cells. $5x10^5$ cells were seeded into 6-well plates; 2 days after, cells were treated with sulindac sulfide for 21 h in the absence or presence of 1 mM or 4mM butyrate. Thereafter, the treated cells were lysed for DEVD-caspase assay. Figure A shows that combination of 1mM butyrate and 50, 100 or 200μM sulfide resulted in synergistic increases of DEVD-caspase activity, that were 126%, 182% and 67% greater, respectively, than the additive effect of the treatments with the reagents alone. Figure B shows that the combination of 4mM butyrate and 50, 100 sulfide resulted in increases of DEVD-caspase activity, which were 5% and 21% greater, respectively, than the additive effect of the treatments with the reagents alone. A combination of 4 mM butyrate and 200 μM sulfide resulted in 39% less DEVD-caspase activity than the expected additive effect of the two reagents. Data are the means of triplicates; error bars indicate the standard deviations. * indicates $p \leq 0.05$, ** $p \leq 0.005$.

activity, that were 5% ($p>0.05$), 21% ($p\leq0.05$) greater, respectively, than the additive effect of the treatments with the reagents alone. DEVD-caspase activity in cells treated with a combination of 4 mM butyrate and 200 μM sulindac sulfide was 39% less than expected from the additive effect of the reagents alone. This is probably the consequence of using optimal concentrations of both reagents.

Butyrate (1 mM) was also synergistic with sulindac sulfone over a range of concentrations (200-600 μM) (Fig 7.9A). A combination of 1 mM butyrate and 200, 300, 400 or 600 μM sulfone resulted in synergistic increases of DEVD-caspase activity, that were 79% ($p\leq0.05$), 128% ($p\leq0.05$), 57% ($p\leq0.05$) and 74% ($p\leq0.005$) greater, respectively, than the additive effect of the treatments with the reagents alone. In contrast to the situation with sulindac sulfide, there was also synergy between 4 mM butyrate and sulfone 200, 300, 400 or 600 μM. A combination of 4 mM butyrate and 200, 300, 400 or 600 μM sulfone resulted in increases of DEVD-caspase activity, that were 42% ($p\leq0.05$), 55% ($p\leq 0.005$), 53% ($p\leq 0.005$) and 32% ($p\leq 0.05$) greater, respectively, than the additive effect of the treatments with the reagents alone (Fig 7.9B).

7.2.5 Combinations of sulindac derivatives and AA (suboptimal concentrations) resulted in synergistic effects on apoptosis.

Sulindac sulfide might induce apoptosis via inhibition of Cox-2, resulting in either decreased levels of prostaglandins (inhibitor of apoptosis) or increased levels of AA (inducer of apoptosis). Alternatively, the effects of sulindac sulfide may be independent of Cox-2 inhibition. The previous results, showing that sulindac sulfone (only a very weak inhibitor of Cox-2) is an effective inducer of apoptosis, suggest that there is a Cox-2-independent mechanism for the induction of apoptosis by NSAIDs.

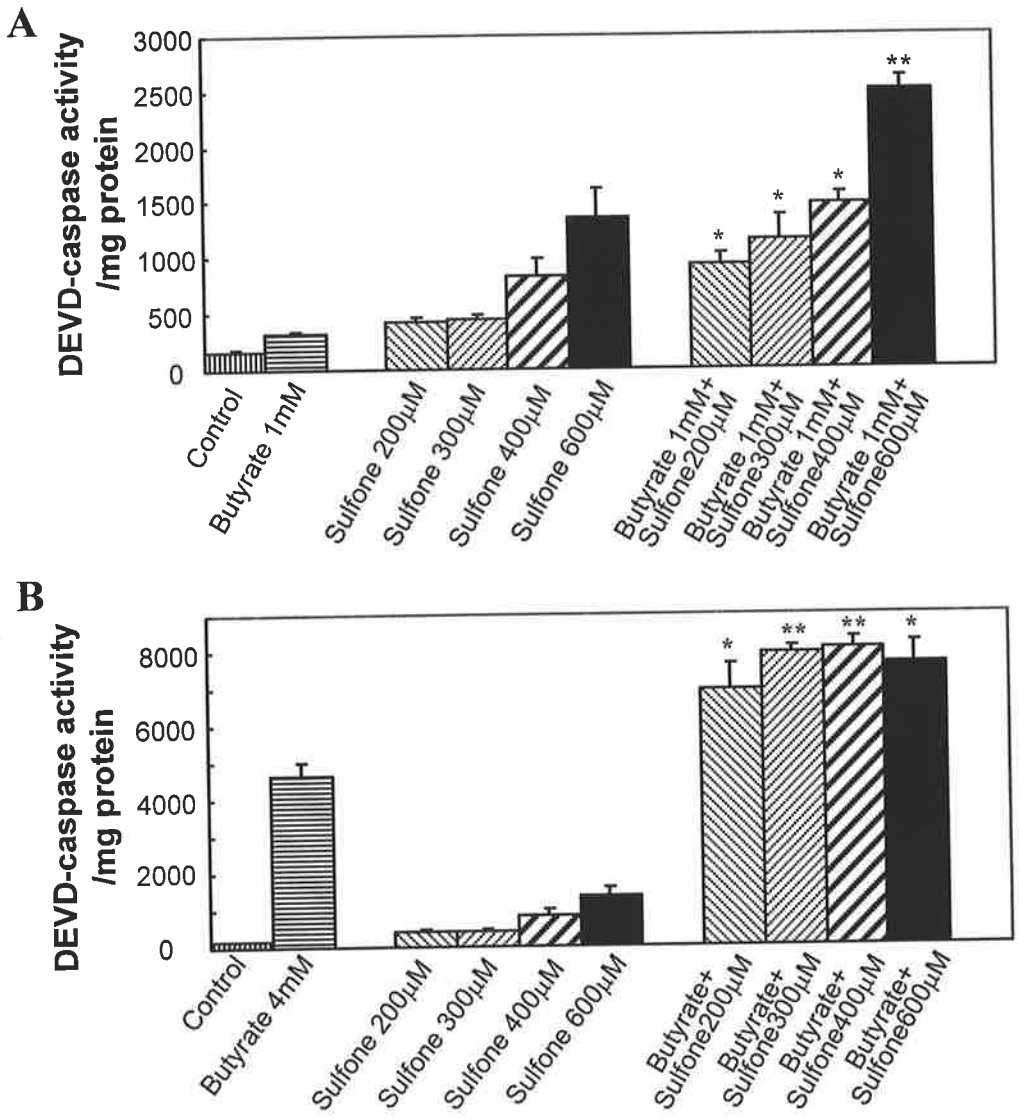


Figure 7.9 Synergistic effects of a combination of 1 mM (A) or 4 mM (B) butyrate and sulindac sulfone on DEVD-caspase activation in LIM1215 cells. $5x10^5$ cells were seeded into 6-well plates; 2 days after, cells were treated with sulindac sulfone for 21 h in the absence or presence of 1mM or 4 mM butyrate. Thereafter, the treated cells were lysed for DEVD-caspase assay. This figure shows that combination of 1mM butyrate and 200, 300, 400 or 600 μM sulfone resulted in synergistic increases of DEVD-caspase activity, that were 79%, 128%, 57% and 74% greater, respectively, than the additive effect of the treatments with the reagents alone. The combination of 4mM butyrate and 200, 300, 400 or 600μM sulfone resulted in increases of DEVD-caspase activity, that were 42%, 55%, 53% and 32% greater, respectively, than the additive effect of the treatments with the reagents alone. Data are the means of triplicates; error bars indicate the standard deviations. * indicates $p \leq 0.05$, ** $p \leq 0.005$.

To investigate this further, the first approach I took was to determine whether AA induces apoptosis as measured by DEVD-caspase activity in LIM1215 cells. Fig 7.10A shows a concentration-dependent effect of AA on DEVD-caspase activity in these cells. 100 μM AA had little effect on induction of DEVD-caspase activity, while 200 μM AA caused a 2.6-fold increase in the activity. DEVD-caspase activity increased even further at 300 μM AA, the highest concentration tested (Fig 7.10A). Therefore, it is possible that sulindac sulfide induces apoptosis by increasing the level of AA in LIM1215 cells.

If sulindac sulfide was simply acting by increasing the levels of AA in cells, an additive (rather than synergistic) interaction on apoptosis would be expected. Therefore, possible additive or synergistic effects between AA and sulindac sulfide were investigated. No synergy was seen between 100 μM AA and 100 μM sulindac sulfide (Fig 7.11A). When the cells were treated with both reagents in combination, there was an increase of DEVD-caspase activity of 46% over the additive effect of the treatments with the reagents alone. However, this was not statistically significant ($p>0.05$). There was a synergistic interaction between 200 μM AA and 100 μM sulindac sulfide (Fig 7.11A). When the cells were treated with both reagents in combination, there was an increase of DEVD-caspase activity of 118% ($p\leq0.05$) over the additive effect of the treatments with the reagents alone.

Furthermore, sulindac sulfone (300-600 μM), which is not expected to increase AA levels, also synergised with AA at both 100 and 200 μM concentrations of the latter (Fig 7.11B). When the cells were treated with a combination of 100 μM AA with 300 or 600 μM sulfone, there were increases of DEVD-caspase activity of 120% ($p\leq0.05$) and 53% ($p\leq0.05$) respectively, over the additive effect of the treatments with the reagents

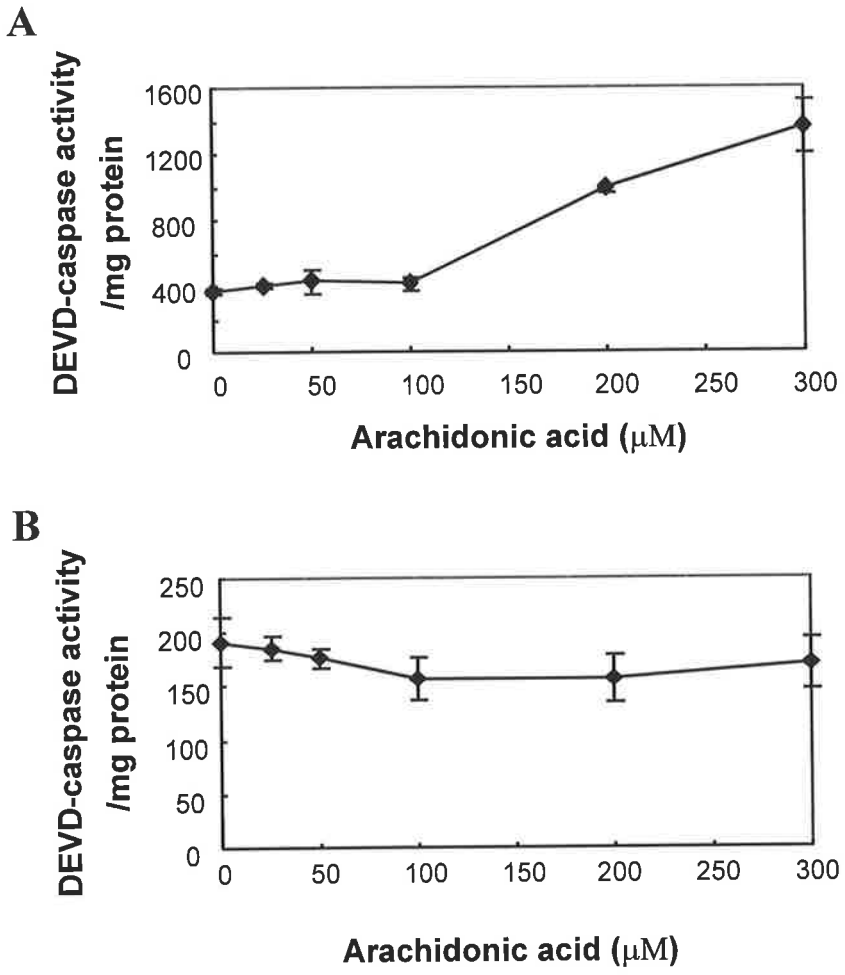


Figure 7.10 DEVD-caspse activity induced by arachidonic acid (AA) in LIM1215 cells (A) or SW480 cells (B). $5x10^5$ cells were seeded into 6-well plates; 2 days after, cells were treated with AA for 21 h. Thereafter, the treated cells were lysed for DEVD-caspase assay. This figure shows that >100 μM AA is able to induce DEVD-caspase activity in LIM1215 cells but not in SW480 cells. Data are the means of triplicates; error bars indicate the standard deviations.

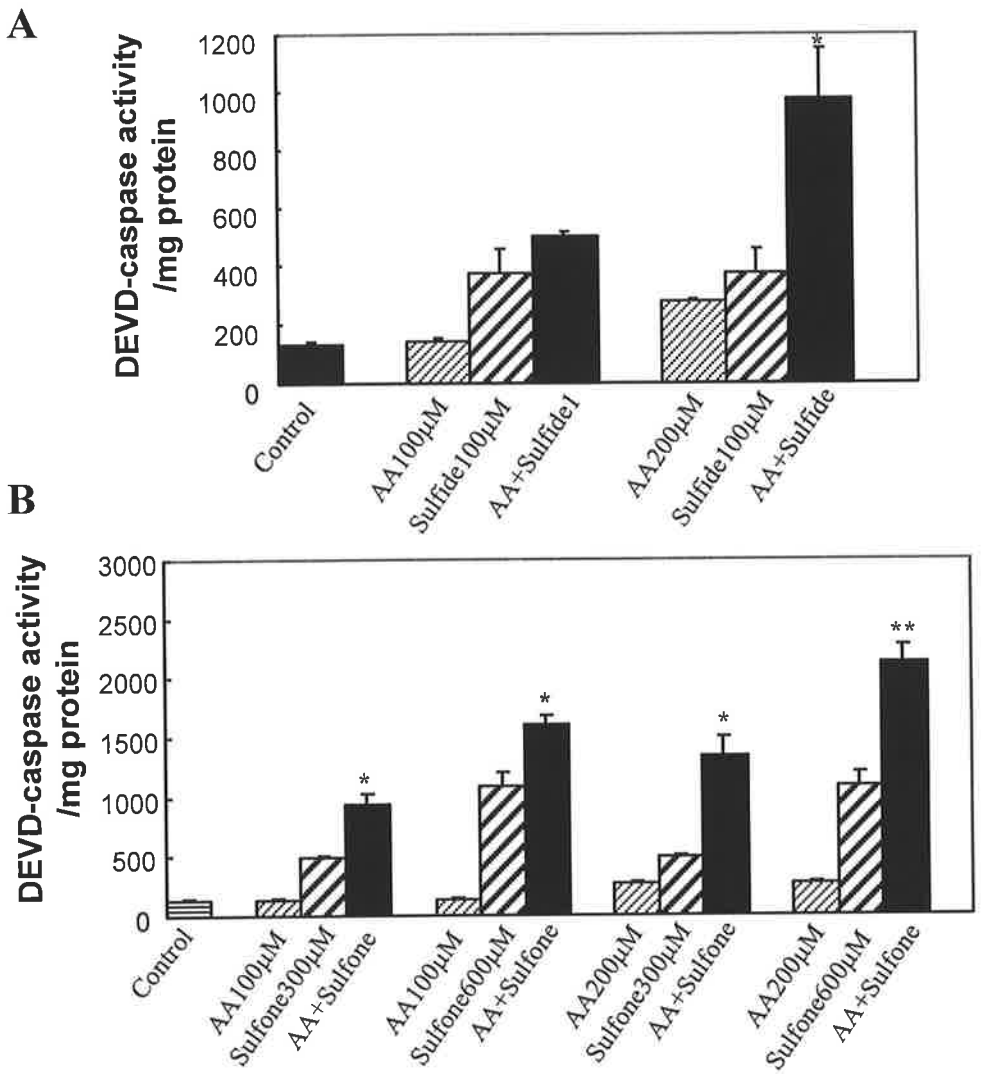


Figure 7.11 The effect of combination of AA and sulindac sulfide (A) or sulfone (B) on LIM 1215 cells. $5x10^5$ cells were seeded into 6-well plates; 2 days after, cells were treated with sulfide or sulfone in the absence or presence of AA for 21 h. Thereafter, the treated cells were lysed for DEVD-caspase assay. This figure (A) shows that combination of 100 μM sulfide and 100 or 200 μM AA resulted in increases of DEVD-caspase activity, that were 46% and 118% greater, respectively, than the additive effect of the treatments with the reagents alone. The combination of 300 μM sulfone and 100 or 200 μM AA resulted in increases of DEVD-caspase activity, that were 120% and 53% greater, respectively, than the additive effect of the treatments with the reagents alone. A combination of 600 μM sulfone and 100 or 200 μM AA (B) resulted in increases of DEVD-caspase activity, that were 145% and 81% greater, respectively, than the additive effect of the treatments with the reagents alone. Data are the means of triplicates; error bars indicate the standard deviations. * indicates $p \leq 0.05$, ** $p \leq 0.005$.

alone. These increases were statistically significant. When the cells were treated with a combination of 200 μM AA with 300 or 600 μM sulfone, there were increases of DEVD-caspase activity of 145% ($p \leq 0.05$) and 81% ($p \leq 0.005$) respectively, over the additive effect of the treatments with the reagents alone. These increases were also statistically significant.

7.2.6 A combination of butyrate and AA (suboptimal concentrations) resulted in synergistic effects on apoptosis.

Next I examined possible synergistic interactions between AA and butyrate. Combinations of 100 μM or 200 μM AA with a sub-optimal concentration (1 mM) of butyrate also resulted in synergistic effects on DEVD-caspase activity (Fig 7.12A). When the cells were treated with a combination of 100 μM or 200 μM AA with 1 mM butyrate, there were increases of DEVD-caspase activity of 18% ($p \leq 0.05$) and 64% ($p \leq 0.05$) respectively, over the additive effect of the treatments with the reagents alone. These increases were also statistically significant. No synergy was seen when an optimal concentration (4 mM) of butyrate was used (Fig 7.12B)

7.2.7 Comparisons in SW480 cells

Some of these experiments were repeated with SW480 cells. Firstly, unlike in LIM1215 cells, AA did not, by itself, increase the DEVD-caspase activity in these cells at any concentrations up to 300 μM (Fig 7.10B). Nor was there any synergy between 100 μM AA and 1 mM butyrate. However, there was synergy between AA and sulfide or sulfone. When the cells were treated with a combination of 100 μM AA and 100 μM sulfide or 300 μM sulfone, there were increases of DEVD-caspase activity that were 280% and 39%

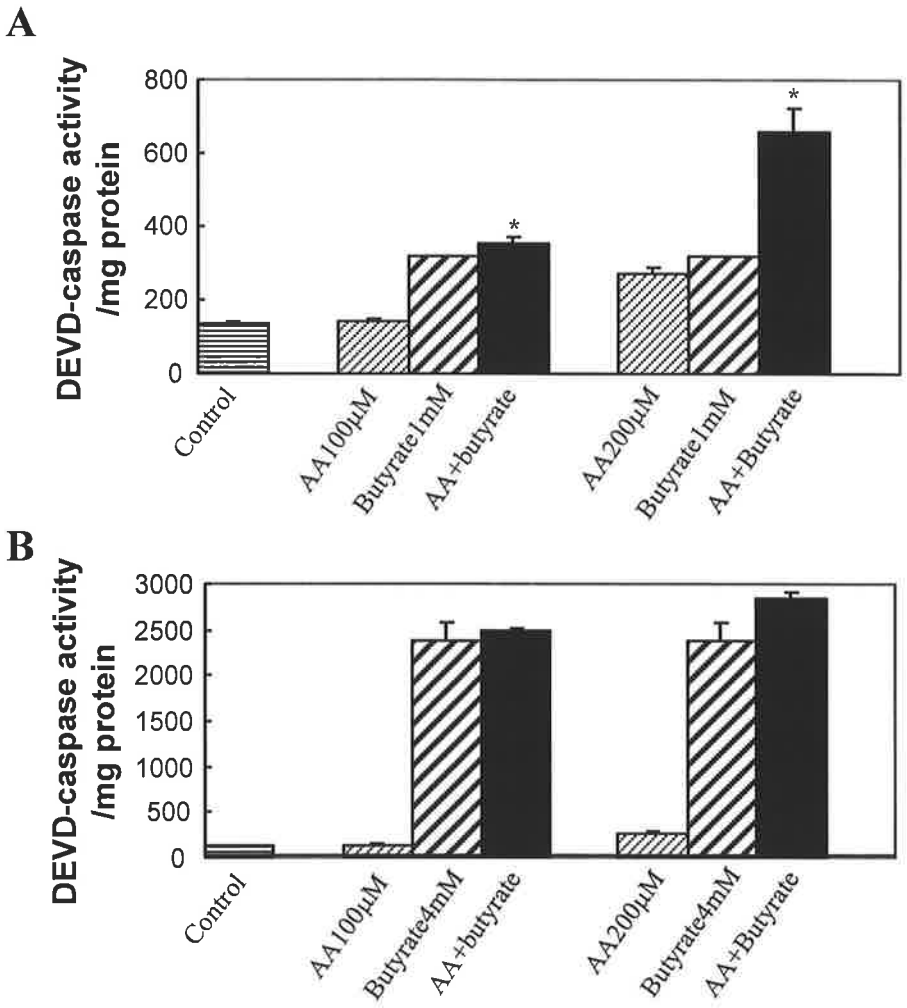


Figure 7.12 The effect of combination of AA and 1 mM (A) or 4 mM (B) butyrate on LIM 1215 cells. $5x10^5$ cells were seeded into 6-well plates; 2 days after, cells were treated with butyrate in the absence or presence of AA for 21 h. Thereafter, the treated cells were lysed for DEVD-caspase assay. This figure (A) shows that combination of 1 mM butyrate and 100 or 200 μM AA resulted in increases of DEVD-caspase activity, that were 18% and 64% greater, respectively, than the additive effect of the treatments with the reagents alone. The combination of 4mM butyrate and 100 or 200 μM AA (B) resulted in increases of DEVD-caspase activity, that were 5% and 14% greater, respectively, than the additive effect of the treatments with the reagents alone. Data are the means of triplicates; error bars indicate the standard deviations. * indicates $p \leq 0.05$, ** $p \leq 0.005$.

greater, respectively, than the control; the additive effect of the treatments with the reagents alone were less than the controls in this experiment (Fig 7.13). These increases were also statistically significant ($p \leq 0.05$).

7.2.8 Butyrate resistant LIM 1215 cells, selected by 3 cycles of 4 mM butyrate treatment, were also resistant to sulindac sulfide and sulfone.

My previous study showed that LIM 1215 cells, selected by 3 cycles of 4 mM butyrate treatment, become insensitive to butyrate. Therefore, one question that needs to be addressed is whether sulindac sulfide and sulfone can overcome this resistance. LIM 1215 cells, selected by 3 cycles of 4 mM butyrate treatment, were further exposed to sulindac sulfide and sulfone for 24 h. The results showed that butyrate-resistant cells were resistant to sulindac sulfide and to sulfone (Fig 7.14). Compared with that of sensitive cells, induction of DEVD-caspase activity by sulindac sulfide and sulfone in resistant cells decreased by 4.5-fold ($p \leq 0.005$) and 3-fold ($p \leq 0.005$), respectively. Compared with resistant cells treated with 4 mM butyrate alone, those treated with 200 μM sulfide alone, or in combination with 4 mM butyrate, had only a small, but significant ($p \leq 0.05$), increase in DEVD-caspase activity (Fig 7.14, middle set of columns). Similar results were seen with 600 μM sulindac sulfone (Fig 7.14, third set of columns). This suggests that there is a common step in the pathways of sulindac- and butyrate-induced apoptosis that is blocked in butyrate-resistant cells.

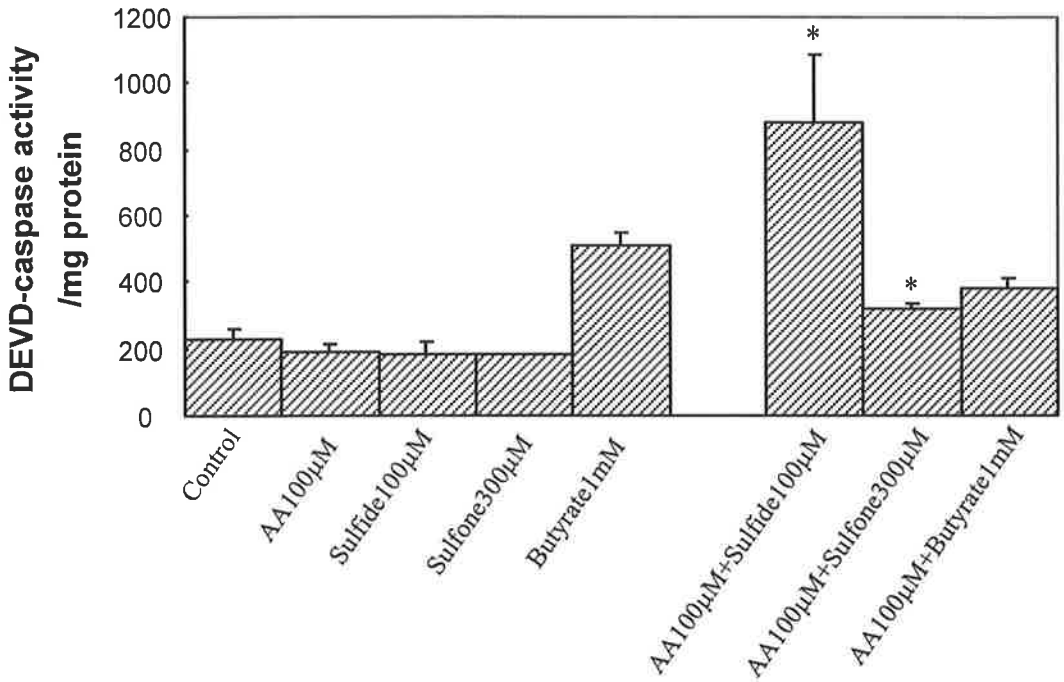


Figure 7.13 Interaction between sulindac derivatives, butyrate and AA in SW480 cells. 5 x10^5 cells were seeded into 6-well plates; 2 days after, cells were treated with sulindac sulfide, sulfone or butyrate for 48 h in the absence or presence of 100 μM AA. Thereafter, the treated cells were lysed for DEVD-caspase assay. This figure shows that 100 μM AA, 100 μM sulfide and 300 μM sulfone alone have no effect on DEVD-caspase activity in SW480 cells. However, a combination of 100 μM AA with 100 μM sulfide or 300 μM sulfone resulted in increases of DEVD-caspase activity, that were 280% and 39% greater, respectively, than the control; the additive effect of the treatments with the reagents alone were less than the controls in this experiment. A combination of 100 μM AA with 1 mM butyrate did not increase DEVD-caspase activity, above that of the additive effect of the treatments with the reagents alone. Data are the means of triplicates; error bars indicate the standard deviations. * indicates $p \leq 0.05$, ** $p \leq 0.005$.

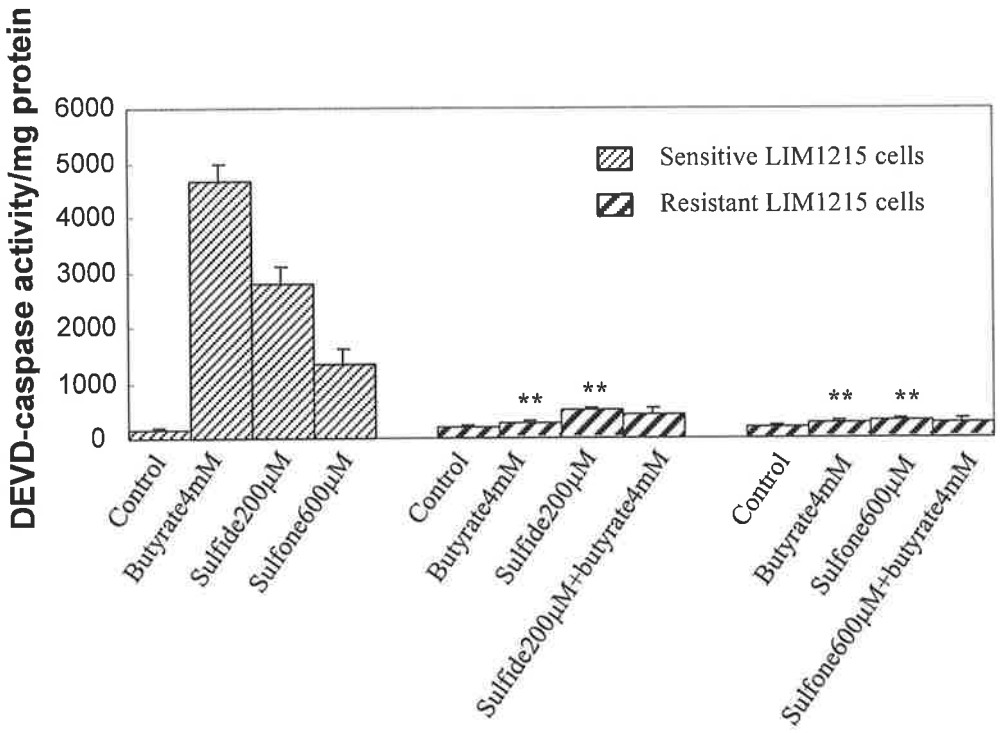


Figure 7.14 The effect of sulindac sulfide and sulfone on butyrate sensitive and resistant LIM1215 cells. $1.5x10^6$cells were seeded into 25cm^2 flasks. Two days after, cells were treated with 4 mM butyrate for 3 cycles; each cycle consisted of 24 h treatment with butyrate, followed by removal of floating apoptotic cells and replacement with fresh medium. After 3 cycles of butyrate treatment, the remaining adherent cells (which were butyrate-resistant) were incubated with 200 μM sulindac sulfide or 600 μM sulfone for a further 24 h, with or without 4 mM butyrate. Compared with that of sensitive cells, induction of DEVD-caspase activity by sulindac sulfide and sulfone in resistant cells decreased by 4.5-fold and 3-fold, respectively. Compared with resistant cells treated with 4 mM butyrate alone, those treated with 200 μM sulfide alone, or in combination with 4 mM butyrate, had only a small increase in DEVD-caspase activity. Similar results were seen with sulindac sulfone. Data are the means of triplicates; error bars indicate the standard deviations. * indicates $p \leq 0.05$, ** $p \leq 0.005$. In this figure, the values for DEVD-caspase activity in butyrate resistant cells (▨) were compared with those for the corresponding concentration of butyrate in the butyrate sensitive cells (▨).

7.2.9 NDGA, a Lox inhibitor, can induce DEVD-caspase activity and potentiate butyrate induced DEVD-caspase activity in sensitive LIM1215 cells but cannot overcome the resistance in butyrate resistant cells.

To further characterise the role of AA metabolites (e.g. HETEs) in apoptosis of colorectal cancer cells, a Lox inhibitor, NDGA was used to test if the Lox pathway was involved in apoptosis. In LIM1215 cells, NDGA induced DEVD-caspase activity in a concentration-dependent way. NDGA began to induce DEVD-caspase activity at 20 μM, while there was a 3.6-fold increase at 40 μM (Fig 7.15A). While NDGA (10 μM) had little effect on induction of DEVD-caspase activity, combining 10 μM NDGA with 1, 2, 3 or 4 mM butyrate, increased DEVD-caspase activity by 59% ($p \leq 0.05$), 112% ($p \leq 0.005$), 72% ($p \leq 0.05$), and 41% ($p \leq 0.05$) respectively, over that induced by butyrate alone (Fig 7.16A). These increases were statistically significant.

To determine whether butyrate resistant cells are also resistant to NDGA, LIM1215 cells were rendered resistant to butyrate by 3 cycles of treatment with 4 mM butyrate, and then treated for a further 24 h with 40 μM NDGA. While in sensitive LIM1215 cells, 40 μM NDGA resulted in a 4-fold increase ($p<0.005$) in DEVD-caspase activity, in resistant cells only a 2-fold increase ($p<0.005$) was evident (Fig 7.17). To determine whether there were any interactions between NDGA and butyrate, sensitive or resistant LIM1215 cells were also treated for 24 h with a combination of 40 μM NDGA and 4 mM butyrate prior to assay of DEVD-caspase activity. NDGA potentiated butyrate-induced caspase-3 activity by 104% ($p \leq 0.005$) in sensitive LIM1215 cells, and by 48% ($p \leq 0.05$) in resistant cells (Fig 7.17). Both of these increases were statistically significant.

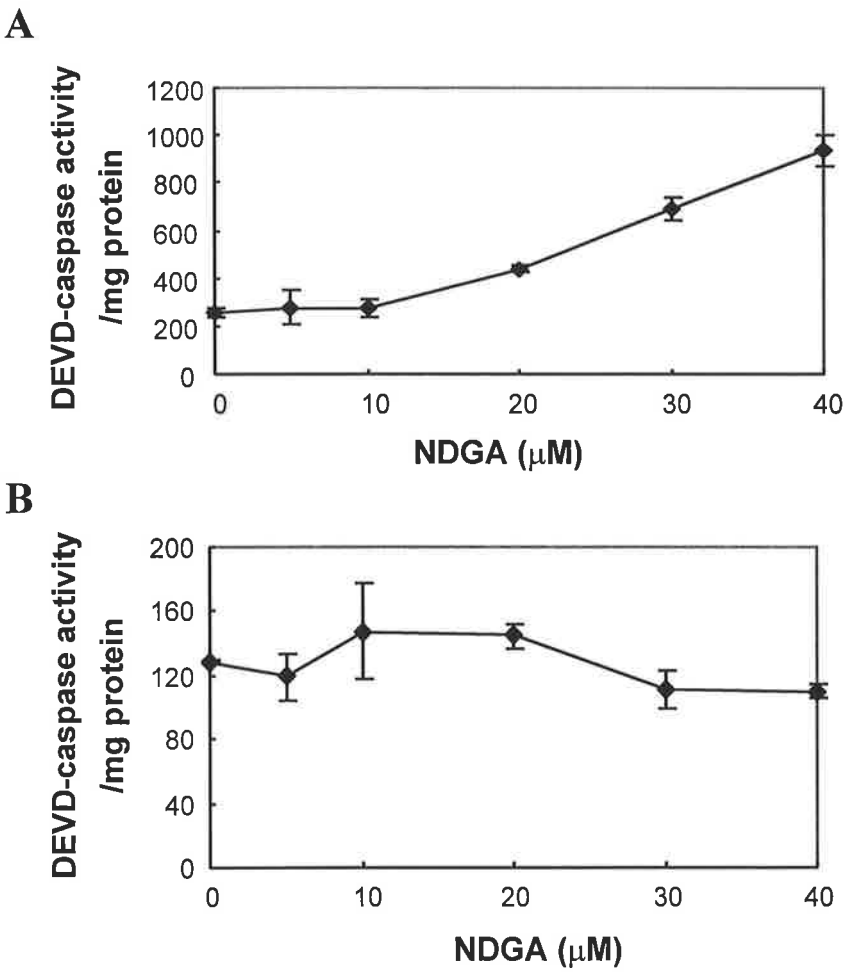


Figure 7.15 DEVD-caspase activity induced by NDGA in LIM1215 cells (A) or SW480 cells (B). $5x10^5$ cells were seeded into 6-well plates; 2 days after, cells were treated with AA for 21 h. Thereafter, the treated cells were lysed for DEVD-caspase assay. This figure (A) shows that in LIM1215 cells DEVD-caspase activity began to increase at 20 μM NDGA, and at 40 μM NDGA there was a 3.6 fold increase compared with control. However, in Sw480 cells (B) no effect of NDGA on DEVD-caspase activity. Data are the means of triplicates; error bars indicate the standard deviations.

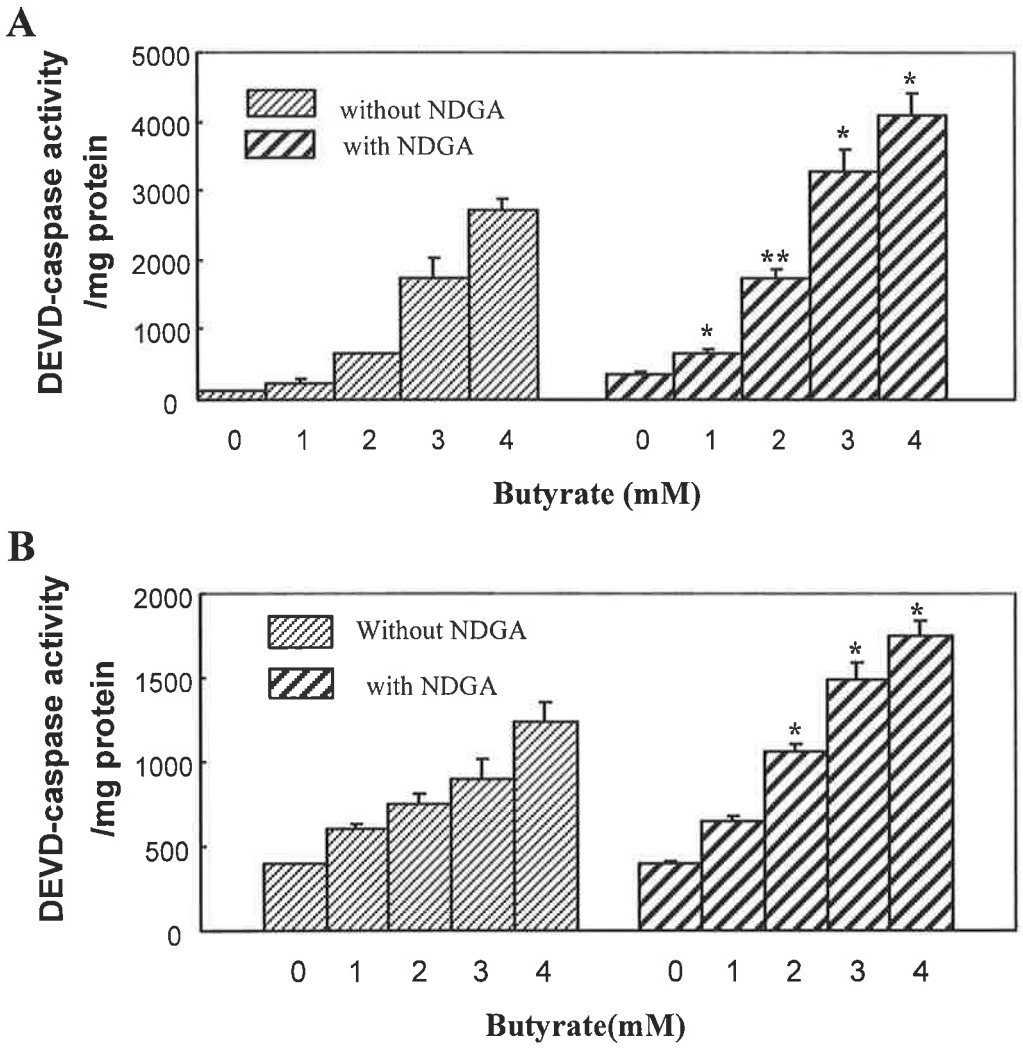


Figure 7.16 Interaction between NDGA and butyrate in induction of DEVD-caspase activity in LIM1215 cells (A) or SW480 cells (B). $5x10^5$ cells were seeded into 6-well plates; 2 days after, cells were treated with butyrate in the absence or presence of NDGA for 24 h. Thereafter, the treated cells were lysed for DEVD-caspase assay. This figure (A) shows that in LIM1215 cells combinations of 1, 2, 3 or 4 mM butyrate and 10 μM NDGA resulted in increases of DEVD-caspase activity, that were 59%, 112%, 72% and 41% greater, respectively, than the additive effect of the treatments with the reagents alone. In SW480 cells (B), these values were 9%, 84%, 112% and 61%, respectively. Data are the means of triplicates; error bars indicate the standard deviations. * indicates $p \leq 0.05$, ** $p \leq 0.005$.

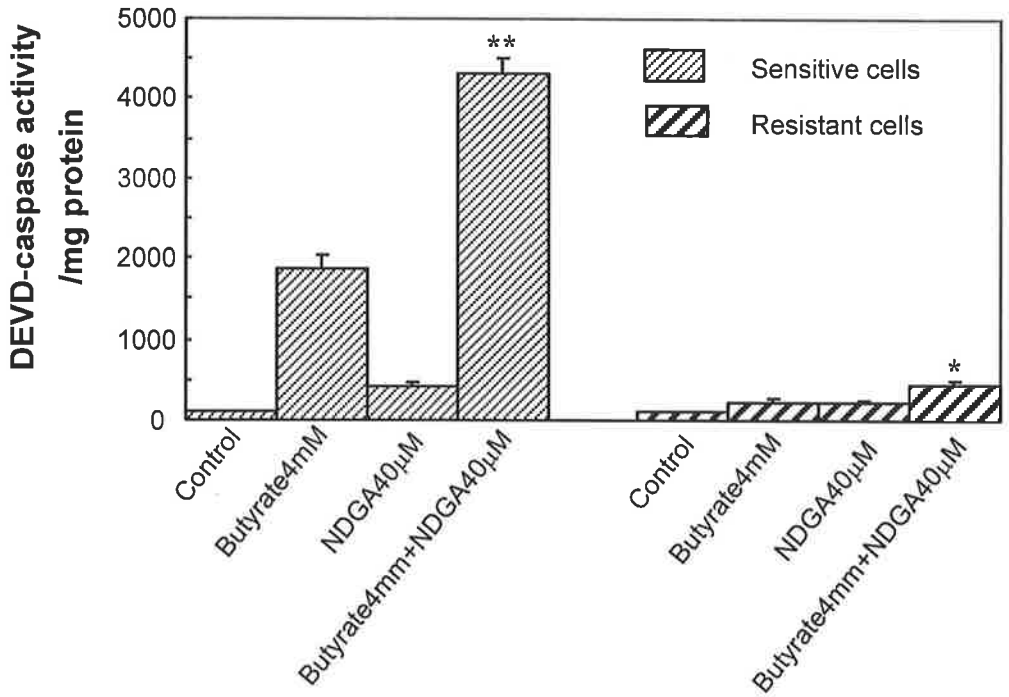


Figure 7.17 The effect of NDGA on butyrate sensitive and resistant LIM1215 cells. $1.5x10^6$cells were seeded into 25 cm^2 flasks. Two days after, cells were treated with 4mM butyrate for 3 cycles; each cycle consisted of 24 h treatment with butyrate, followed by removal of floating apoptotic cells and replacement with fresh medium. After 3 cycles of butyrate treatment, the remaining adherent cells (which were butyrate-resistant) were incubated with 40 μM NDGA for a further 24 h, with or without 4 mM butyrate. In sensitive cells, NDGA resulted in a 4-fold increase in DEVD-caspase activity and potentiated 4 mM butyrate-induced DEVD-caspase activity by 104%. In resistant cells, NDGA resulted in a 2-fold increase and potentiated 4 mM butyrate-induced DEVD-caspase activty by 48%. Data are the means of triplicates; error bars indicate the standard deviations. * indicates $p \leq 0.05$, ** $p \leq 0.005$.

These results suggest that NDGA does not overcome butyrate resistance but does potentiate butyrate-induced apoptosis.

Some experiments were repeated in SW480 cells. As for AA, these cells were also resistant to NDGA-induced DEVD-caspase activation (Fig 7.15B). However, NDGA was able to enhance DEVD-caspase activity induced by varying concentrations of butyrate (1-4 mM). When the cells were treated with a combination of 10 μM NDGA and 1, 2, 3 or 4 mM butyrate, there were increases of DEVD-caspase activity of 9%, 84%, 112% and 61%, respectively, over the additive effects of the treatments with the reagents alone (Fig 7.16B). Apart from the 9% increase seen with 10 μM NDGA and 1 mM butyrate together, the other increases were statistically significant ($p \leq 0.05$). By contrast, there was no potentiation of sulindac sulfide-induced caspase activation by NDGA, at any concentrations between 10 and 160 μM (highest concentration tested) (Fig 7.18).

7.3 DISCUSSION

The experiments described in this chapter address two issues, firstly, the mechanisms mediating butyrate-induced apoptosis and resistance to butyrate and secondly, possible synergistic interactions between butyrate and Cox/Lox inhibitors in induction of apoptosis of CRC cells.

Previous studies have shown that butyrate up-regulates both Cox-2 (Crew *et al.*, 2000) and Lox (Kamitani *et al.*, 1998) in CRC cells. One implication of this is that either Cox-2 and/or Lox mediates induction of apoptosis by butyrate. Alternatively, the upregulation of Cox-2 and/or Lox may be a protective mechanism helping to suppress the butyrate-induced apoptosis, and therefore prolonging the survival of the cells. To further investigate this, inhibitors of Cox-2 and Lox were used. If either Cox-2 or Lox are

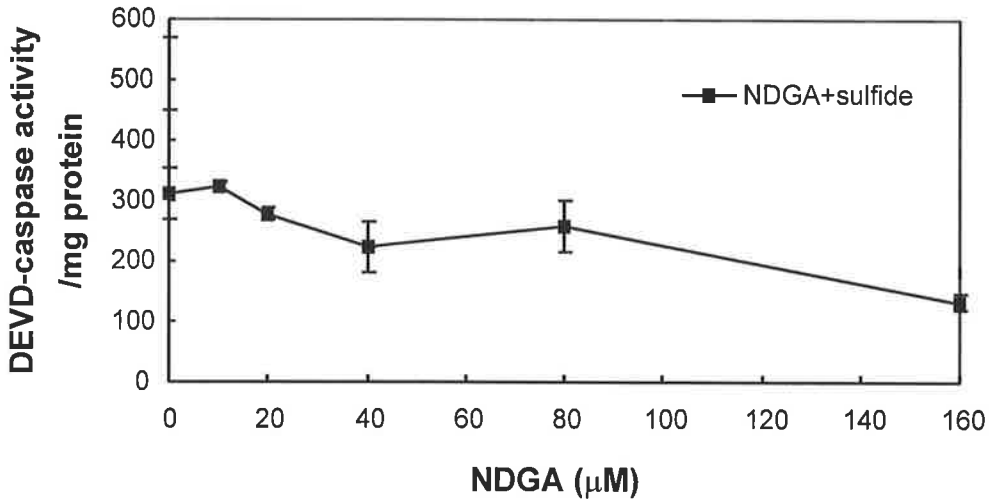


Figure 7.18 Interaction between NDGA and sulindac sulfide in induction of DEVD-caspase activity in SW480 cells. $5x10^5$ cells were seeded into 6-well plates; 2 days after, cells were treated with NDGA, in the presence of 100 μM sulindac sulfide for 48 h. Thereafter, the treated cells were lysed for DEVD-caspase assay. This figure shows that NDGA was not able to potentiate sulindac sulfide-induced DEVD-caspase activity in SW480 cells. Data are the means of triplicates; error bars indicate the standard deviations.

essential for butyrate-induced caspase activation and apoptosis, then inhibitors of these enzymes should block these effects. On the other hand, if Cox-2 or Lox protect cells from apoptosis and are important in resistance of these cells to butyrate, then inhibitors of these enzymes should overcome the resistance to butyrate. The results showed that both types of inhibitors, sulindac sulfide and NDGA, enhanced butyrate-induced apoptosis in both LIM1215 and SW480 cells. This is consistent with a role for both Cox-2 and Lox in the resistance of these cells to butyrate, although it does not prove this hypothesis.

Both Cox-2 and Lox decrease levels of AA in the cells, the former by converting AA to prostaglandins and the latter by converting AA to HETEs. AA is thought to be important for apoptosis of colorectal cancer cells, by stimulating the conversion of sphingomyelin to ceramide, a known mediator of apoptosis (Chan *et al.*, 1998). The studies reported in this chapter confirm this finding in LIM1215 cells. Furthermore, I have shown for the first time that AA was able to induce the activation of caspase-3 in LIM1215 cells, although two very recent studies have now shown this also in human retinoblastoma Y79 cells (Vento *et al.*, 2000) and in cultured spinal cord neurons (Garrido *et al.*, 2000). In view of these findings, Cox and Lox may protect against butyrate-induced apoptosis (i.e. induce butyrate resistance) by decreasing the levels of AA in the cells. It is not clear whether the concentrations of AA used in this study cause changes in intracellular levels of AA that are physiological.

However, there are three pieces of evidence against a role for Cox-2 and/or AA in the mechanism of resistance to butyrate. Firstly, the interaction between sulindac sulfide and AA on activation of caspase-3 was synergistic rather than additive. This was also reported in the study by Chan, et al. (1998). Synergy between these agents would not be

expected if sulindac sulfide was simply acting via increase in the intracellular levels of AA. Secondly, sulindac sulfone does not inhibit Cox-2 (Piazza *et al.*, 1997; Thompson *et al.*, 1997), but was able to induce caspase-3 activity and apoptosis in LIM1215 cells; although higher concentrations and longer incubation time were required for sulindac sulfone than for sulfide. Thirdly, SW480 cells lack Cox-2 (Smith *et al.*, 2000), but still undergo apoptosis and can be rendered butyrate-resistant. Therefore, there are likely to be other mechanisms present. Recently, it was reported (He *et al.*, 1999) that sulindac sulfide and sulfone inhibit the activity of peroxisome proliferator- activated receptor δ (PPARδ). Furthermore, the concentrations of sulindac sulfone required to inhibit PPARδ activity were higher than those for sulindac sulfide as observed in my studies for effects on apoptosis. Also, the effective concentrations of sulfide for inhibition of PPARδ activity (He *et al.*, 1999) were similar to those affecting apoptosis in my experiments. PPARδ is a transcription factor, belonging to the same family of steroid/thyroid hormone nuclear receptors (Kastner *et al.*, 1995; Mangelsdorf *et al.*, 1995). The tumour suppressor APC, which is often mutated in familial adenomatous polyposis, also down regulates the transcriptional activity of PPARδ (He *et al.*, 1999). It may be that one or more of the genes regulated by PPARδ is a gene whose product suppresses apoptosis in colorectal cancer cells and that by blocking PPARδ, sulindac enhances butyrate-induced apoptosis. The relevance of this pathway to mechanisms of butyrate induced apoptosis and butyrate resistance now requires further investigation.

The second issue addressed in these studies concerns potential synergistic interactions between butyrate and Cox-2/Lox inhibitors in apoptosis of colorectal cancer cells. The results here showed that suboptimal butyrate (1 mM) was synergistic with

sulindac over a range of concentrations of sulfide (50-200 μM) and sulfone (200-600 μM), in effects on induction of DEVD-caspase activity. This may be important in chemoprevention of colorectal cancers where a combination of sulindac and high dietary fibre may yield better effects than either agent alone. Both sulindac sulfide and sulfone have antitumour activity. It is likely that this activity is due to enhanced apoptosis of the tumour cells. In LIM1215 cells, there was an inverse correlation between growth inhibition and DEVD-caspase activation following treatment with sulindac derivatives.

The synergy between butyrate and sulindac also suggests that the mechanism of butyrate-induced apoptosis is different from that induced by sulindac sulfide and sulfone. The kinetics of butyrate-induced apoptosis was slower than that of sulfide but faster than that of sulfone. Although others have shown that sulindac sulfide and sulfone induce apoptosis by morphological criteria, this study is the only one, to my knowledge, to examine their effects on caspase activity. However, the roles of caspases in sulindac-induced apoptosis remain unclear. Despite the complete inhibition of sulfide- and sulfone-induced DEVD-caspase activity by two types of caspase peptide inhibitors, there was no inhibition of DNA fragmentation. This may mean that caspase-independent pathways are primarily involved.

Since recent studies have shown that butyrate induces Cox-2 expression (Crew *et al.*, 2000) and that over-expression of Cox-2 in rat intestinal epithelial cells causes them to become resistant to butyrate-induced apoptosis (Tsujii and DuBois, 1995), Cox-2 may be important also in the resistance to butyrate induced by treatment of LIM1215 cells with 3 cycles of 4 mM butyrate treatment (see chapter 4). This raises a question. Is up-regulation of Cox-2 expression a factor in the resistance to apoptosis in our cell model? If Cox-2 is

involved in this resistance, Cox inhibitor sulindac sulfide should overcome this resistance. The results showed that sulindac sulfide only weakly overcame the resistance. This suggests that Cox expression is not likely to be an important factor in butyrate resistance in LIM1215 cells.

Other evidence has suggested that Lox-catalyzed products have a profound influence on the development and progression of human cancer (Steele *et al.*, 1999) as well as regulating cell survival and apoptosis in rat Walker W256 carcinosarcoma cells (Tang *et al.*, 1996). To determine whether Lox is involved in butyrate resistance, Lox inhibitor NDGA was used to treat butyrate-sensitive and -resistant LIM 1215 cells. The results showed that NDGA was able to potentiate modestly butyrate-induced DEVD-caspase activity in sensitive and resistant LIM1215 cells but did not overcome the butyrate resistance. Therefore, Lox is, only a minor factor in the resistance to butyrate in these cells.

The reason for the failure of both NDGA and AA to induce apoptosis in SW480 cells is not known, but this is in keeping with the reduced response of these cells to butyrate, sulindac sulfide and sulindac sulfone. These cells lack expression of Cox-2 (Smith *et al.*, 2000). This is unlikely to explain their resistance to NDGA and AA, since neither of these two agents act via Cox-2. Furthermore, SW480 cells are still sensitive to butyrate and sulindac sulfide and sulfone although less than LIM1215 cells. It is not known whether they lack Lox, and this needs further investigation. One important difference between LIM1215 cells and SW480 cells is that LIM1215 cells have wild type p53 (Agapova *et al.*, 1996) while SW480 cells have mutant p53 in which arg_{273} is changed to his and pro_{309} is changed to ser (Abarzua *et al.*, 1995). The observation that AA still

synergised with sulindac sulfide and sulfone in causing apoptosis of SW480 cells suggests that AA does something to SW480 cells (ie. sensitize them to another inducer of apoptosis) without inducing the full process of apoptosis, itself. NDGA did not sensitise SW480 cells to sulindac sulfide and sulfone although it did sensitise them to butyrate. A further complicating issue is that AA can induce morphological changes of apoptosis (Chan *et al.*, 1998) without apparently inducing caspase-3 activation. Clearly, the interactions between these agents are complex and require further studies.

The cross resistance of butyrate-resistant LIM1215 cells to sulindac sulfide and sulfone may indicate that there is a common step in the pathways of sulindac- and butyrate-induced apoptosis, that is blocked in butyrate-resistant cells. This has clinical relevance, since in cases of colorectal cancer where the tumour has acquired resistance to butyrate, sulindac derivatives are likely to be clinically ineffective. Further studies are required to elucidate the nature of the block to apoptosis in butyrate-resistant cells and to identify chemotherapeutic agents that can overcome the resistance.

CHAPTER 8

THE INTERACTION BETWEEN BUTYRATE AND ZINC CHELATOR (TPEN) ON APOPTOSIS OF COLORECTAL CANCER CELLS

8.1 INTRODUCTION

One previous study has suggested that levels of the group II dietary metal zinc are approximately 2 fold higher in human colon cancers than in adjacent normal tissue (Song *et al.*, 1993). However, this is controversial since one other study has reported that zinc is reduced in colorectal cancer cells compared with normal tissue (Xiao and Henderson, 1992). Further studies by Song et al (Song *et al.*, 1995) suggested that zinc is required for upregulation of the inducible form of prostaglandin synthase, and that maintaining an optimal zinc nutriture is important for normal prostaglandin synthesis of colonocytes. Since PGE_2 significantly increased the rate of zinc uptake of colonic tumor cells (Song *et al.*, 1995), abnormalities in PGE_2 synthesis in colonic tumor cells may result in an abnormal zinc status of these cells.

Zinc, an important trace element, is mainly present in vivo in two ways: tightly bound pools within zinc proteins, which constitute a largely fixed pool of cellular Zinc (associated with metalloenzymes and zinc finger proteins) as well as labile zinc pools, which are loosely associated with proteins, lipids, cytoskeletal processes or sequestered in vesicles. These labile pools, which are believed to be important in cytoprotection and the regulation of apoptosis, are readily influenced by zinc deprivation or supplementation (Vallee and Falchuk, 1993; Zalewski *et al.*, 1993).

In zinc deficiency, there is not only damage to the epithelial tissues including lesions in the skin and gastrointestinal epithelium (Solomons, 1988), but also a heightened susceptibility of the epithelium to damage by other toxins (e.g. in the duodenum by colchicine (Dinsdale and Williams, 1977). One of the mechanisms of zinc deficiency-

induced epithelial damage is by increased death of epithelial cells via the cell suicide process of apoptosis (gene-directed cell death) (Zalewski and Forbes, 1993). Zinc deficiency can also result in cell death by necrosis, especially if the apoptotic pathway is dysfunctional (Kolenko *et al.*, 1999). The mechanisms by which zinc deficiency results in enhanced apoptosis remain unclear.

The first evidence that zinc may be important in the regulation of apoptosis came from a study in 1977 by Elmes (1977), who reported greatly increased frequencies of apoptotic cells in the small intestinal crypts of zinc-deficient rats. She proposed that this apoptosis was triggered by a failure of DNA synthesis in these cells. Subsequent studies by the same author and by others showed that the frequency of apoptotic cells was also markedly increased in many tissues of adult animals, as well as in the neuroepithelium in foetal rats borne by zinc-deficient dams (Zalewski and Forbes, 1993). The extent to which apoptosis occurs in zinc-deficient humans is less clear, due to the unavailability of tissues and organs. Increased apoptosis in zinc-deficient humans has been inferred from studies of peripheral leukocytes of patients with Down's syndrome and may also account for the characteristic vesicular skin lesions and severe T cell depletion in zinc malabsorption syndromes such as acrodermatitis enteropathica (Solomons, 1988).

Increased apoptosis in vivo could either be a direct consequence of a decrease in intracellular zinc in the affected cells or secondary to changes in other tissues. There is at least one example of the latter, where apoptosis in the thymus of zinc-deficient rodents was at least in part due to excessive levels of circulating glucocorticoids triggered by a zinc deficiency-associated stress response (Fraker and Telford, 1997). This appears to be an exception, however, and evidence from in vitro studies indicates that apoptosis can

result directly from a decline in intracellular zinc induced by culture of cells in either zinc-free medium or in the presence of membrane-permeant Zinc chelators, such as TPEN.

Zinc is now recognised as one of the factors that regulate caspase-3 activation (Chai *et al.*, 1999). TPEN is an inducer of caspase-3 activation in various types of cells, while zinc suppresses caspase-3 activation both in intact cells and cell-free extracts (Aiuchi *et al.*, 1998; Perry *et al.*, 1997). The mechanism by which zinc acts is not known, but caspase-6 has been reported to be highly sensitive to zinc (Stennicke and Salvesen, 1997; Takahashi *et al.*, 1996). Since caspase-6 has been implicated in processing of the caspase-3 precursor (Srinivasula *et al.*, 1996), zinc may affect this step.

There have been no previous studies of the regulation of apoptosis by zinc in colon cancer cells. In this study, TPEN was used to deplete intracellular zinc in LIM1215 and SW480 colorectal cancer cells. The effects on activation of caspase-3 and caspase-6, as well as the cleavage of p21, were determined, both in the presence and absence of butyrate. The levels of labile pools of intracellular zinc were monitored using a UV-excitable intracellular zinc(II)-specific fluorophore Zinquin (Zalewski *et al.*, 1993).

8.2 RESULTS

8.2.1 Intracellular zinc depletion by TPEN as demonstrated by decrease in Zinquin fluorescence

In order to deplete intracellular zinc, LIM1215 cells were treated for 1 h with 25 μM TPEN (a membrane-permeable zinc chelator). Control cells were treated with DMSO alone (0.5%), the solvent for TPEN. Intracellular zinc content (specifically the more labile pools of zinc) was monitored by loading the cells with the UV-excitable zinc-specific

fluorophore Zinquin. There was no UV autofluorescence of the cells in the absence of Zinquin (not shown). Fig 8.1C shows the fluorescence of the control cells (no TPEN). Note the largely cytoplasmic fluorescence indicating labile zinc in the cytoplasm of these cells. Fig 8.1D shows the strong quenching of Zinquin fluorescence by TPEN. TPEN has a much higher affinity for zinc than Zinquin (Zalewski *et al.*, 1993). Thus, in LIM1215 cells specific mean fluorescence intensity decreased from 13.96 + 7.72 pixels (n=200) in cells incubated with Zinquin alone, to 8.51 + 3.4 pixels (n=200) in cells incubated with Zinquin plus 25 μM TPEN ($p \leq 0.005$).

8.2.2 Zinc depletion induces apoptosis, DNA fragmentation and caspase-3 activation in colorectal cancer cell lines

To determine the effects of zinc depletion on apoptosis of LIM1215 cells, four markers of apoptosis were used: - morphological analysis, flow cytometric analysis with propidium iodide, DNA fragmentation and caspase-3 activation. LIM1215 cells were treated with 25 μM TPEN for 4 h. Fig 8.2 shows the blebbing of the TPEN-treated cells and formation of apoptotic bodies (B), as well as fragmentation of chromatin (D). By contrast, control cells had normal morphology (A) and intact chromatin (C).

Next, TPEN-treated LIM1215 cells were analysed for apoptosis by flow cytometry with propidium iodide. LIM1215 cells were treated with 25μM TPEN for up to 4 h. Fig 8.3 shows a progressive increase in apoptotic cells, as indicated by appearance of an increasing fraction of cells with less than 2N DNA content, preceding the G_1 peak on the fluorescence scans. Cells containing hypodiploid DNA increased 1 h after addition of TPEN and plateaued at 3 h. Cell lysates were prepared and assayed for DNA fragmentation (Fig 8.4) and DEVD-caspase activity (Fig 8.5). The first increase in DNA

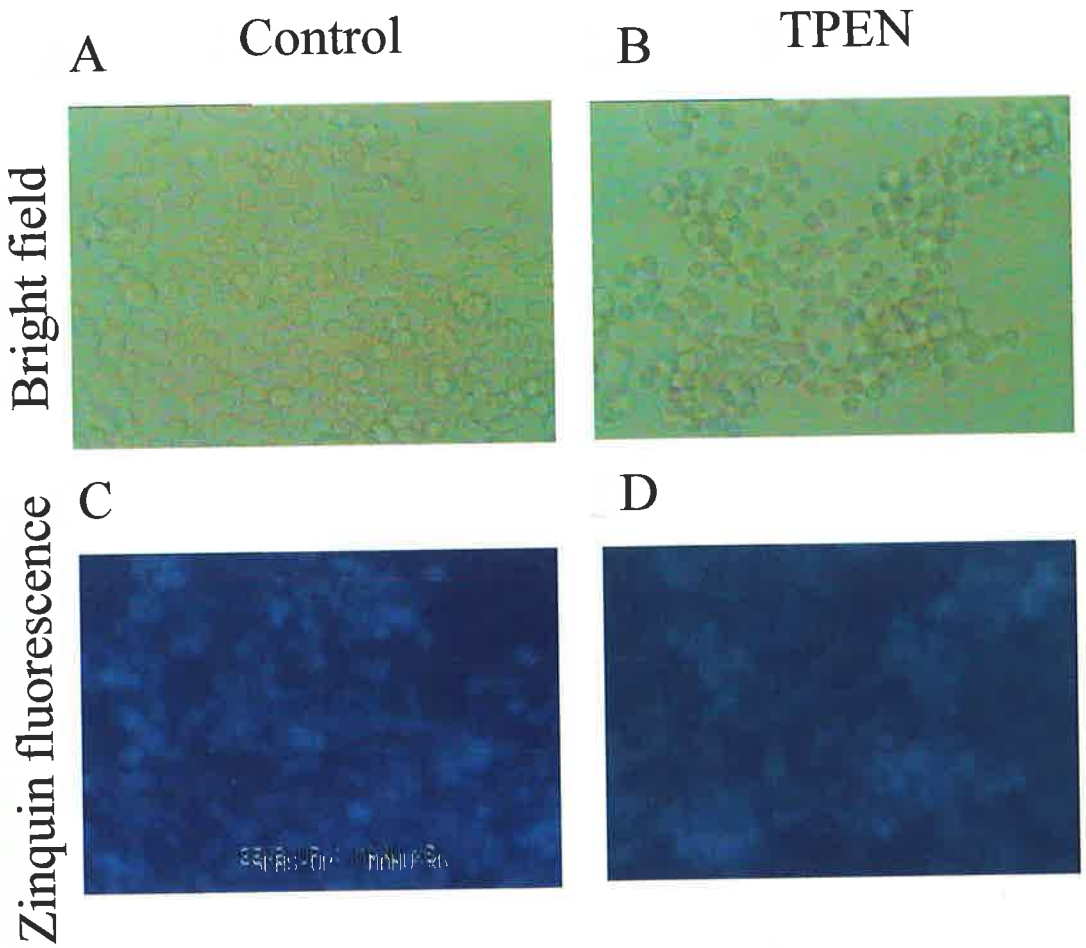


Figure 8.1 Zinc depletion by short-term (1 h) treatment with TPEN in LIM1215 cells. Coverslip monolayers of LIM1215 cells were treated for 1 h in the presence of control solvent DMSO (0.5%) or 25μM TPEN. Cells were washed with HBSS (3X) before addition of 25μM Zinquin for a further 30 min. Coverslips were inverted on to microscope slides and examined under UV epifluorescence. Images were captured and stored using a video camera and image analysis software. Fluorescence intensity was quantified in 200 cells from ten different fields. Mean fluorescence intensity was calculated.

A & B: Bright field images of typical fields for control (A) and TPEN-treated cells (B). Overall magnification(500x)

C& D: Zinquin fluorescence images of typical fields for control (C) and TPEN-treated cells (D). Initial magnification(500x)

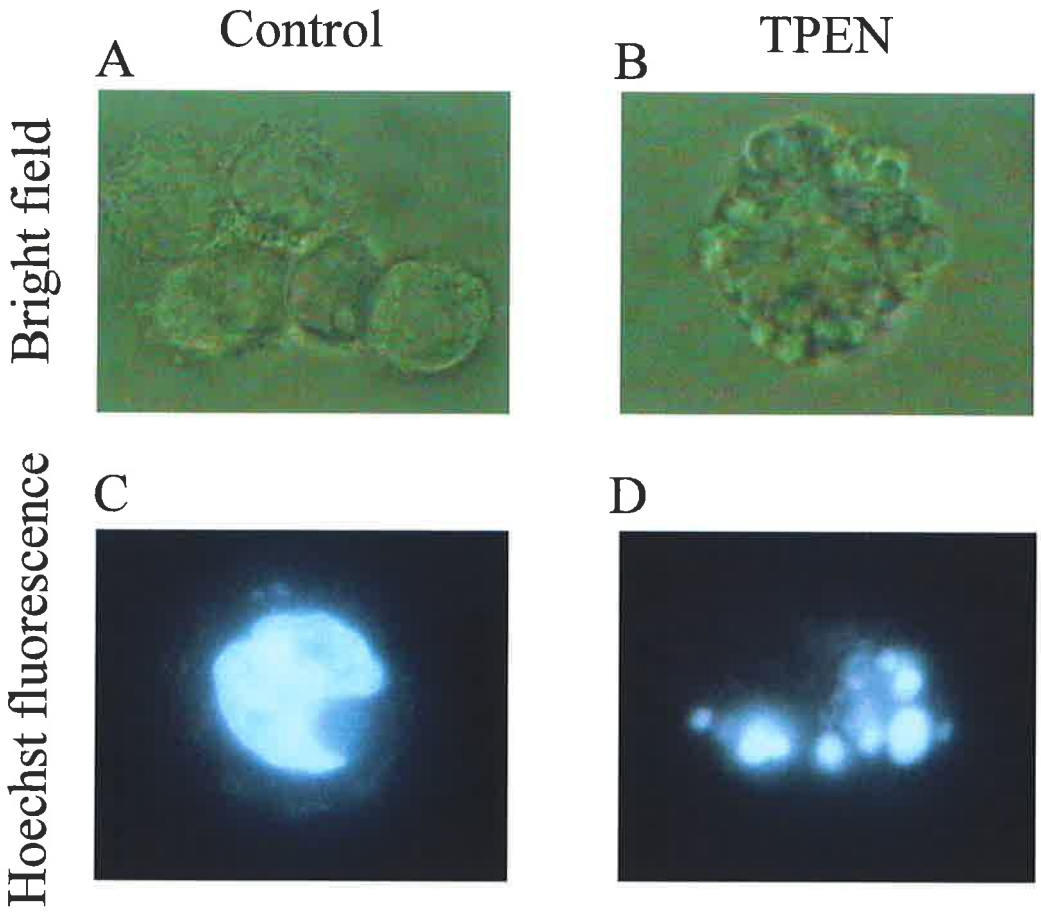


Figure 8.2 Apoptotic morphology of LIM1215 cells treated for 4 h with TPEN. $7x10^5$ LIM1215 cells were seeded into wells and, after 2 days, treated for 4 h with control solvent DMSO (0.5%) or 25μM TPEN. Cells were washed with HBSS (3X) before addition of 110μg/ml Hoechst dye 33342 for a further 5 min to stain the chromatin. Cells were examined under bright field illumination or UV epifluorescence. Images were captured and stored using a video camera and image analysis software.

A & B: Bright field illumination of typical cells are shown for control (A) and TPEN-treated cells (B). Overall magnification (2500x). Note the blebbing of the cell and formation of apoptotic bodies in B.

C& D: UV epifluorescence of typical cells are shown for control (C) and TPEN-treated cells (D). Initial magnification (2500x). Note the fragmentation of chromatin in D.

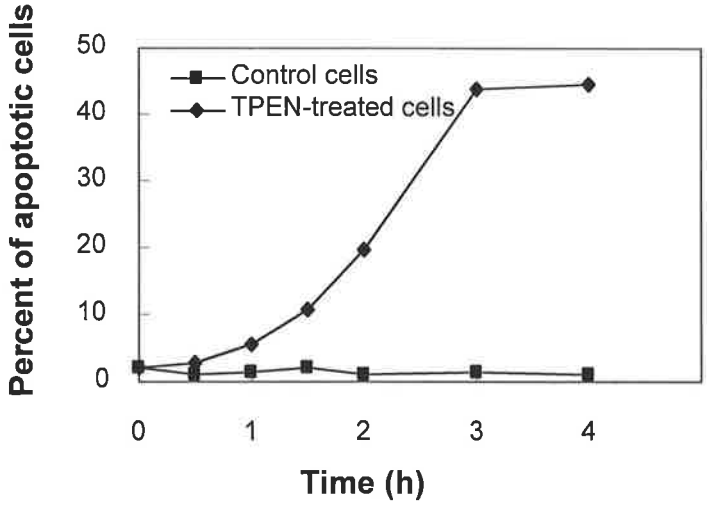


Figure 8.3 Flow cytometric analysis of apoptosis in LIM1215 cells following treatment with TPEN. TPEN (25μM) was added at time 0 and, at 1 h intervals, combined non-adherent and adherent cell populations were washed, fixed and analyzed by flow cytometery (see Methods). "sub-G1" apoptotic cells were determined for control cells (filled squares) and for TPEN-treated cells (filled rhombuses). Graph shows increase in apoptotic cells, beginning at 0.5 h and increasing up to 3 h.

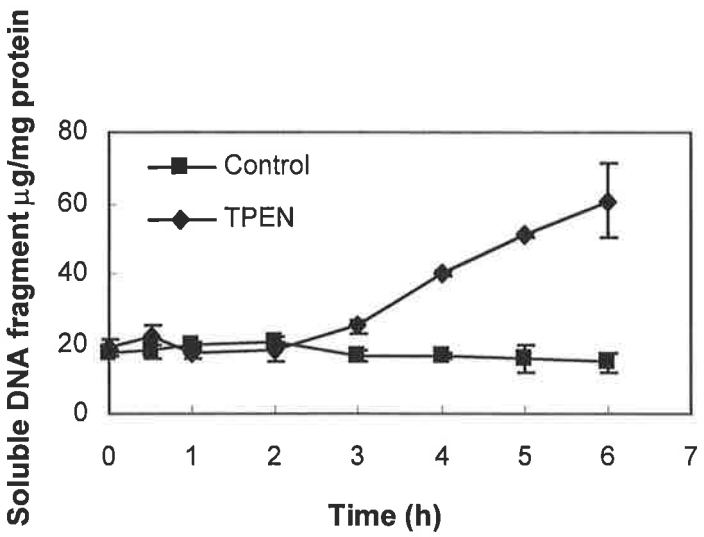


Figure 8.4 Time-course of DNA fragmentation induced by TPEN in LIM 1215 cells. $8x10^5$ cells LIM 1215 cells were seeded into 6-plate wells. 2 days after, cells were treated with 25μM TPEN for 0-6 h, and then were harvested for DNA fragmentation. This graph shows that induction of DNA fragmentation begins at 2 h after addition of TPEN and then increases thereafter. Error bars indicate standard deviations (n=3). A typical graph is shown.

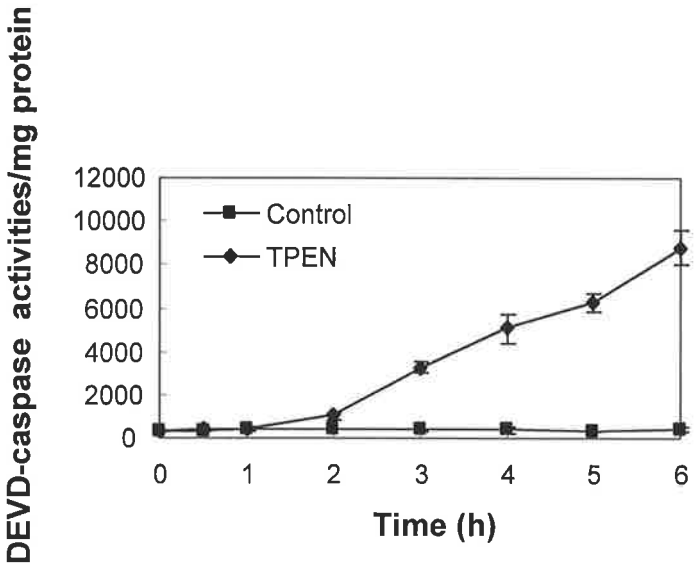


Figure 8.5 Time-course of DEVD-caspase activity induced by TPEN in LIM 1215 cells. $8x10^5$ cells LIM 1215 cells were seeded into 6-plate wells. 2 days after, cells were treated with 25μM TPEN for 0-6 h, and then were harvested for DEVD-caspase activity. This graph shows that induction of DEVD-caspase begins at 2 h after addition of TPEN and then increases linearily. Error bars indicate standard deviations (n=3). A typical graph is shown.

fragmentation occurred after 2 h and a large increase occurred after 3 h and was almost linear up to 6 h (Fig 8.4). A similar time course was seen for DEVD-caspase activation induced by TPEN (Fig 8.5). DEVD-caspase activity increased slowly between 1 and 2 h after addition of TPEN (Fig 8.5). After 2 h, there were sharp rises in DEVD-caspase activity, increasing linearly up to 6 h (the latest time point tested).

The fluorogenic substrate assay, showing an increase in DEVD-caspase activity in TPEN-treated cells, could indicate rises in caspase-3 or caspase-7, both of which can cleave this substrate. The western blot in Fig 8.6 confirmed that TPEN induced caspase-3 activity, as determined by the decrease in the 32KDa zymogen form (CPP32) of caspase-3. The antibody used in this blot failed to detect the 17 and 12KDa cleaved products. Others have noted this as well (Aiuchi *et al.*, 1998). The decrease in CPP32 was most evident between 2 and 3 h after addition of TPEN (Fig 8.6), consistent with the rapid onset of DEVD-caspase activity (Fig 8.5). CPP32 completely disappeared by 3 h. DNA fragmentation only began to increase rapidly 3 h after addition of TPEN, consistent with this being a downstream event following caspase-3 activation.

Fig 8.7 shows concentration dependence curves for both DEVD-caspase activity and DNA fragmentation. There was a small, significant ($p \leq 0.05$) increase in DEVD-caspase activity with 6.25 μM TPEN, followed by highly significant increases ($p \leq 0.005$) at 12.5 μM and 25 μM TPEN (the latter yielding a maximal response). Similar results were found for DNA fragmentation (Fig 8.7). To confirm these findings in another colorectal cancer cell line, SW480 cells were used. Similarly, both DNA fragmentation and DEVD-caspase activity began to increase with 6.25 μM TPEN and maximal response was achieved with 25 or 50 μM TPEN (Fig 8.8). As for responses to butyrate and

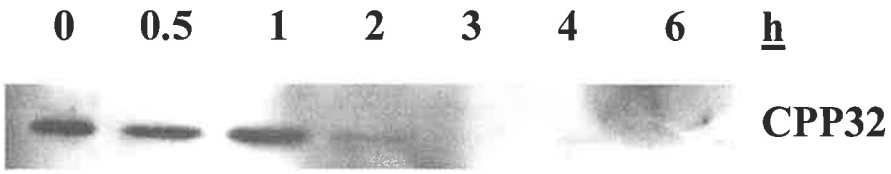


Figure 8.6 Western blot showing the activation of caspase-3 in TPEN-treated LIM1215 cells. $1.5x10^6$ cells were seeded into 25 cm^2 flasks. Two days after, cells were were treated with 25 μM TPEN. At the times indicated, the cells were collected for determination of caspase-3 activation by Western blotting. Equal amount of protein was loaded in each well. The antibody used in the study detects the 32KDa precursor (CPP32) of caspase-3 but not the cleaved 17 and 12KDa sub-units. Caspase-3 activation is indicated by disappearance of CPP32 band. This figure shows that caspase-3 was first activated at 2 h after addition of TPEN. CPP32 completely disappeared by 3 h.

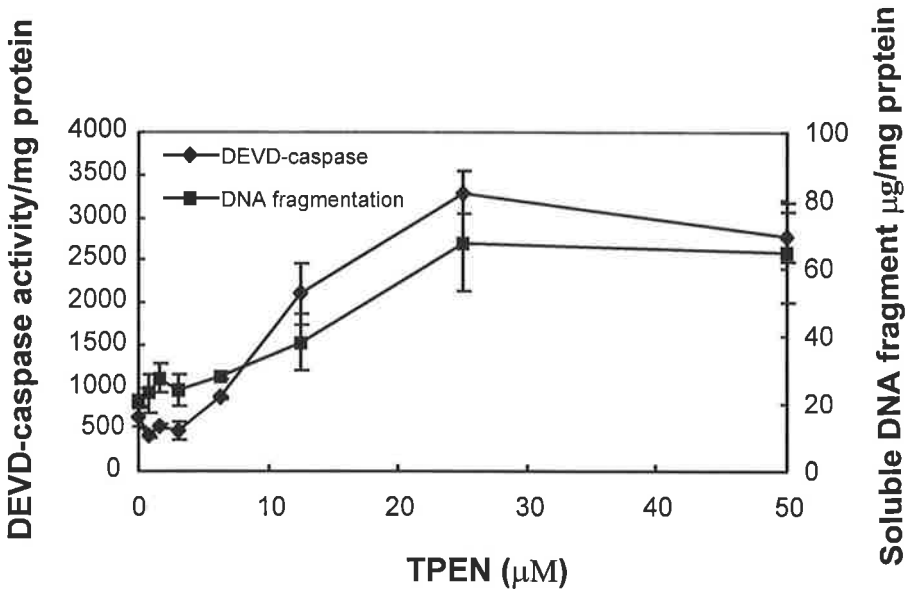


Figure 8.7 DEVD-caspase activity and DNA fragmentation induced by TPEN in LIM 1215 cells. $8x10^5$ LIM 1215 cells were seeded into 6-well plates. Two days after, cells were treated with different concentrations of TPEN for 3 h and then were harvested for DEVD-caspase activity (◆) and DNA fragmentation (■). This graph shows that the induction of DEVD-caspase activity and DNA fragmentation began at 6.25μM TPEN, and was maximal at 25μM TPEN. Error bars indicate standard deviations (n=3). A typical graph is shown.

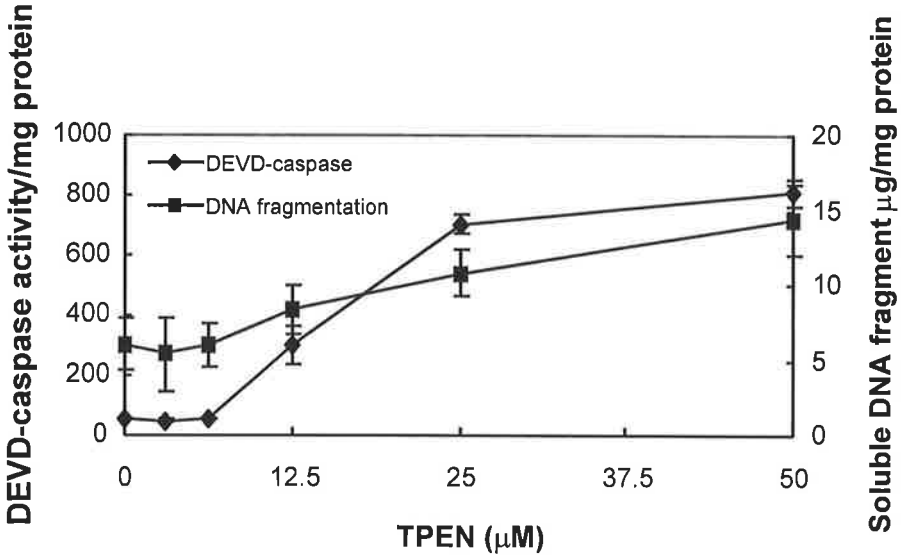


Figure 8.8 DEVD-caspase activity and DNA fragmentation induced by TPEN in SW480 cells. $5x10^5$ SW480 cells were seeded into 6-well plates. Two days after, cells were treated with different concentrations of TPEN for 3 h and then were harvested for DEVD-caspase activity (◆) and DNA fragmentation (■). This graph shows that the induction of DEVD-caspase activity and DNA fragmentation began at 6.25μM TPEN, and was maximal at 25μM TPEN. Error bars indicate standard deviations (n=3). A typical graph is shown.

sulindac sulfide and sulfone, apoptotic response to TPEN was lower in SW480 cells and LIM1215 cells.

8.2.3 Zinc supplementation blocks TPEN-induced apoptosis.

Since TPEN not only chelates zinc but also copper and nickel, it is possible that some of the effects of TPEN are due to chelation of these other metal ions. To test this, cells were treated with TPEN in the presence or absence of zinc sulphate. The induction of DEVD-caspase activity by TPEN was prevented at all time points, when cells also received exogenous $ZnSO_4$ (25-200 μM) (Fig 8.9). Thus, after 5 h of TPEN treatment in LIM1215 cells, there was a 17-fold increase in DEVD-caspase activity over the control (no TPEN). This increase was almost completely suppressed when cells were treated with TPEN in the presence of 25, 50, 100 or 200 μM $ZnSO_4$. The reversal studies with exogenous zinc support the hypothesis that TPEN is acting by chelating zinc.

8.2.4 Zinc depletion by TPEN induces caspase-6 activation

To further investigate the mechanism of caspase-3 activation in TPEN-treated cells, caspase-6, which is known to cleave CPP32 to its active form (Srinivasula *et al.*, 1996), was assayed. LIM1215 cells were treated with 25 μM TPEN for varying periods of time. Caspase-6 activity was monitored by Western blotting to detect the active p20 subunit or by a fluorogenic substrate assay with VEID-AFC. The active p20 subunit of caspase-6 did not increase significantly in the first 2 h after addition of TPEN to LIM1215 cells, but increased steadily after 2 h (Fig 8.10). Similar results were obtained by the enzymatic assay, with increases in VEID-caspase occurring after 2 h in LIM1215 cells (Fig 8.11).

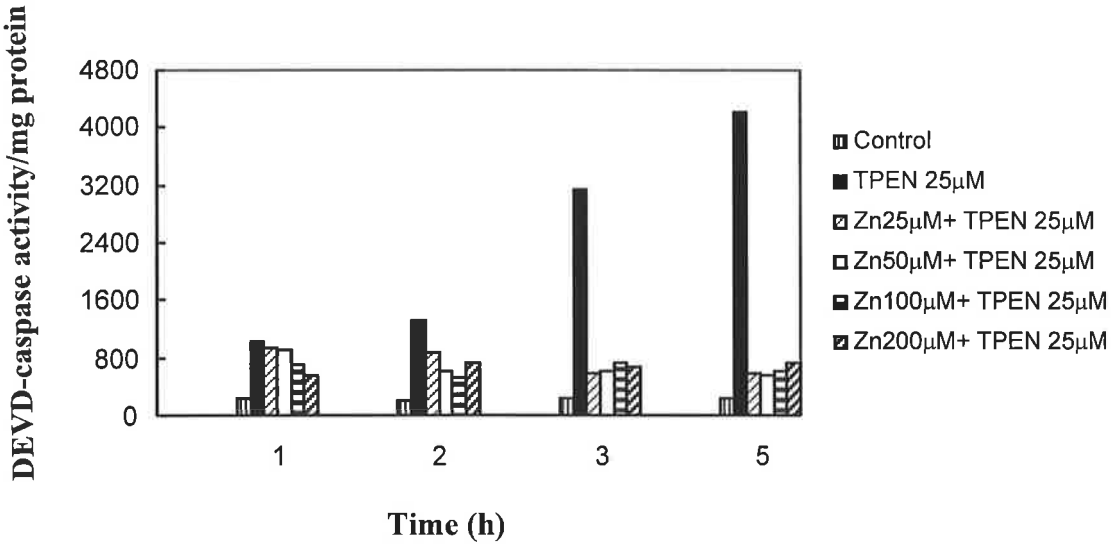


Figure 8.9 Inhibition of the activation of TPEN-induced DEVD-caspase by zinc in LIM 1215 cells. $8x10^5$ cells were seeded into $25cm^2$ flasks. Two days after, $ZnSO_4$ 25-200 μM was added to the cells for 1 h before addition of 25 μM TPEN. Cells were incubated for a further 1-5 h. This graph shows that zinc (25 μM or higher) inhibits the activation of DEVD-caspase induced by 25 μM TPEN.

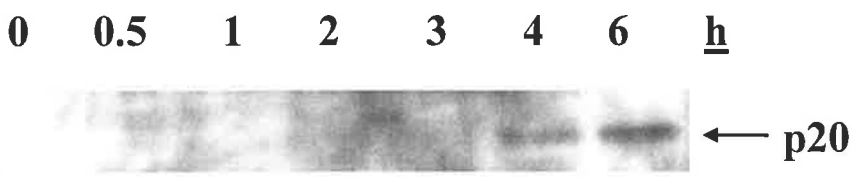


Figure 8.10 Western blot showing the activation of caspase-6 in TPEN-treated LIM1215 cells. $1.5x10^6$ cells were seeded into 25 cm^2 flasks. Two days after, cells were were treated with 25μM TPEN. At the times indicated, the treated cells were collected for determination of caspase-6 by Western blotting. Equal amount of protein was loaded in each well. This figure shows that caspase-6 was activated at 2-3 h after addition of TPEN. The active 20 kDa p20 sub-unit gradually increased thereafter.

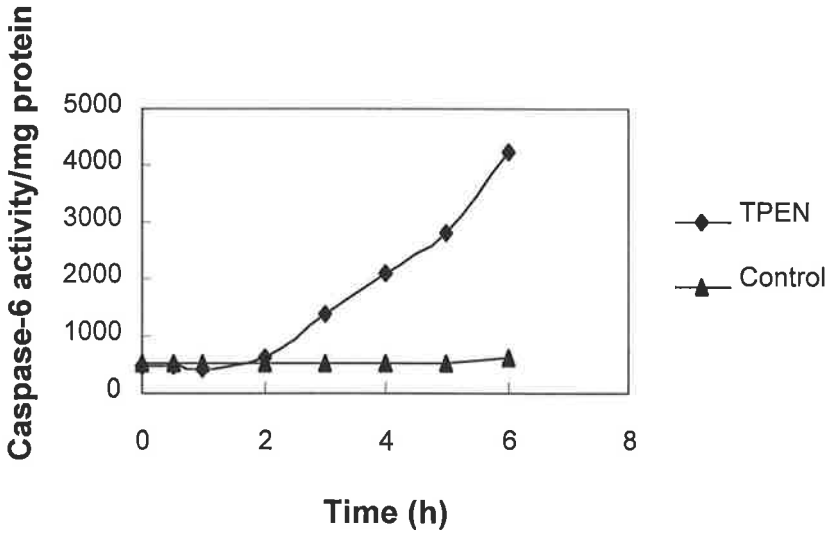


Figure 8.11 Time course of caspase-6 activity induced by TPEN in LIM 1215 cells. $8x10^5$ cells were seeded into 6-plate wells. Two days after, cells were treated with 25 μM TPEN for 0-6 h, and were harvested for VEID-caspase activity. This graph shows that induction of VEID-caspase first occurs at 2 h after addition of TPEN (filled rhombuses) and then gradually increases. There was no increase in cells incubated without TPEN (filled triangles). Data represent mean of duplicates.

8.2.5 Caspase inhibitors block TPEN-induced caspase-3 activation but only partially block DNA fragmentation

To determine whether caspases are involved in the subsequent apoptosis of TPEN-treated colorectal cancer cells, two specific membrane-permeable peptide inhibitors of caspase-3 were used, zDEVD-fmk and zVAD-fmk. Both inhibitors completely inhibited DEVD-caspase activity in TPEN-treated LIM1215 cells (Fig 8.12 A), although it was not determined whether the peptide inhibitors were blocking the active caspase-3 or rather the caspase-dependent steps leading to activation of caspase-3. Despite the complete inhibition of DEVD-caspase activity, there was only partial inhibition of DNA fragmentation, even at higher concentrations of inhibitors (Fig 8.12 B). Inhibition of DNA fragmentation by 25 μM zVAD-fmk was 51% ($p \leq 0.05$) and by 10 μM zDEVD-fmk was 34% ($p \leq 0.05$).

8.2.6 Interaction between TPEN and butyrate in LIM1215 cells

In order to investigate if there are additive or synergistic effects of TPEN and butyrate on apoptosis of colorectal cancer cells, LIM1215 cells were treated with sub-optimal and optimal concentrations of butyrate for 18 h, and then TPEN was added for a further 2 h. Butyrate (1, 2 or 4 mM) was synergistic with 12.5 μM TPEN (Fig 8.13). For example, a combination of TPEN with 1, 2 or 4 mM butyrate resulted in synergistic increases of DEVD-caspase activity, that were 82% ($p \leq 0.05$), 57% ($p \leq 0.05$) or 28% ($p \leq 0.05$), greater, respectively, than the additive effect of the treatments with the reagents alone. However, there was no synergy between TPEN and 0.5 mM butyrate (Fig 8.13). Similarly, there was synergy between 1-4 mM butyrate and 12.5 μM TPEN in induction of DNA fragmentation in LIM1215 cells (Fig 8.14).

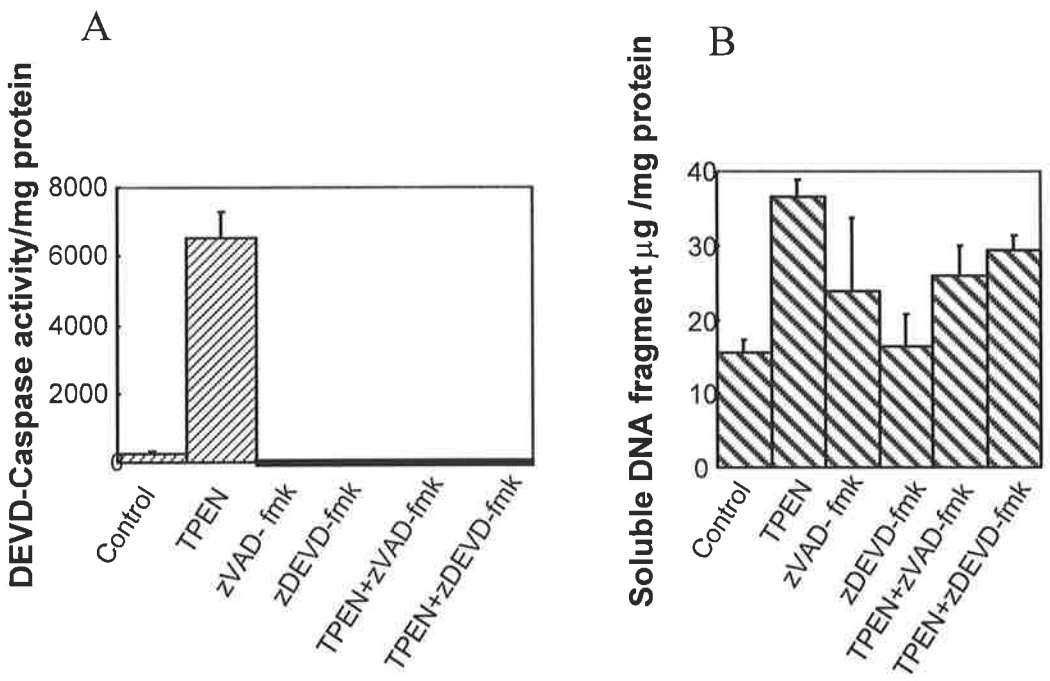


Figure 8.12 Effects of caspase peptide inhibitors on DEVD-caspase activity and DNA fragmentation. LIM1215 cells were pretreated for 1 h with 10µM zDEVD-fmk or 25µM zVAD-fmk before addition of 25 µM TPEN (hatched columns) for a further 3.5 h. Cell lysates were prepared and assayed for DEVD-caspase activity (A) or soluble DNA fragments (B). This graph shows that peptide inhibitors completely inhibit the activity of DEVD-caspase (A) but only partially inhibit DNA fragmentation (B). Soluble DNA fragments in the cells treated with zVAD-fmk and zDEVD-fmk decreased by 51% and 34%, respectively($p<0.05$). Data are means of triplicates from a typical experiment; error bars indicate standard deviations.

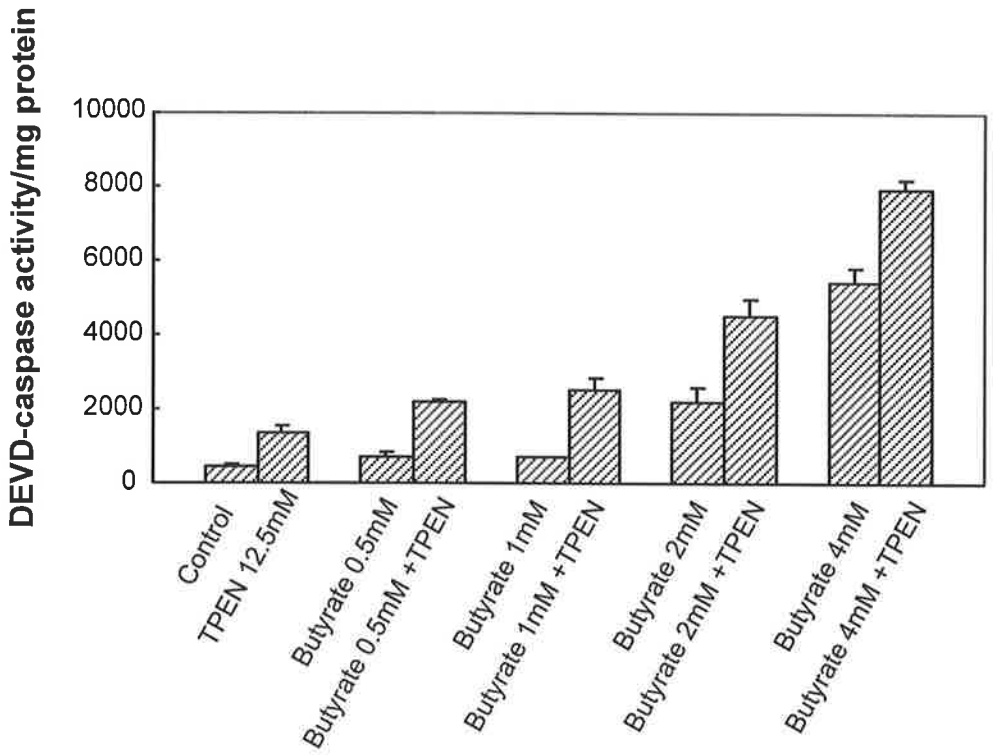


Figure 8.13 Synergistic interaction between butyrate and TPEN in induction of DEVD-caspase activity in LIM 1215 cells. $7x10^5$ cells were seeded into 6-well plates. Two days after, the cells were primed with 0.5, 1, 2 or 4mM butyrate for 18 h and then incubated with 12.5 μM TPEN for a further 2 h and collected for DEVD-caspase activity. This figure shows that combination of TPEN with 1, 2 or 4 mM butyrate resulted in synergistic increases of DEVD-caspase activity, that were 82% ($p \leq 0.05$), 57% ($p \leq 0.05$) or 28% ($p \leq 0.05$), greater, respectively, than the additive effect of the treatments with the reagents alone. However, a combination of TPEN with 0.5 mM butyrate resulted in an increase of DEVD-caspase activity, that was 49% greater than the additive effect of the treatments with the reagents alone (not statistically significant, $p=0.07$). Error bars indicate standard deviations (n=3). A typical graph is shown.

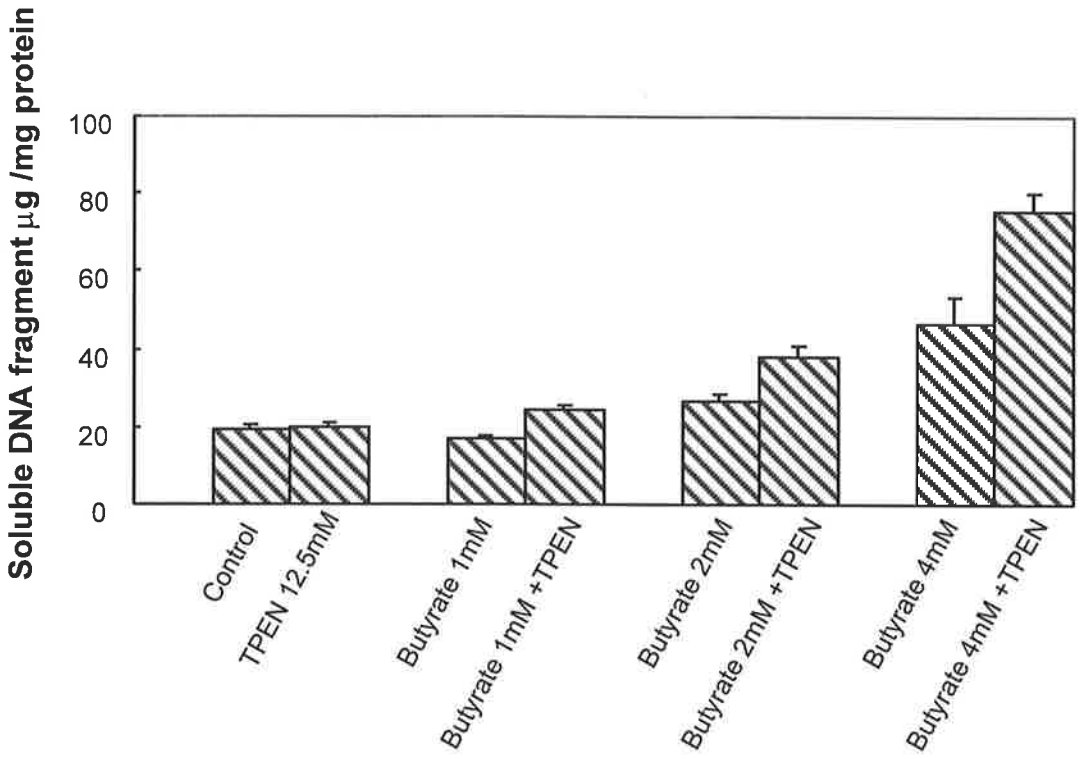


Figure 8.14 Synergistic interaction between butyrate and TPEN in induction of DNA fragmentation in LIM 1215 cells. $7x10^5$ cells were seeded into 6-well plates. Two days after, the cells were primed with 1, 2 or 4mM butyrate for 18 h and then incubated with 12.5 µM TPEN for a further 2 h and assayed for DNA fragmentation. This figure shows that combination of TPEN with 1, 2 or 4mM butyrate resulted in synergistic increases of DNA fragmentation, that were 744% ($p \leq 0.005$), 125% ($p \leq 0.005$) or 101% ($p \leq 0.005$), greater, respectively, than the additive effect of the treatments with the reagents alone. Error bars indicate standard deviations (n=3). A typical graph is shown.

The above results show that depleting cellular zinc enhances butyrate-induced apoptosis of LIM1215 cells. In order to determine whether the opposite holds, LIM1215 cells were treated with supplemental zinc sulphate (12.5-200 μM), in the presence or absence of zinc ionophore pyrithione (1 μM), before the addition of 4mM butyrate. DEVD-caspase activity was measured. Fig 8.15 shows that zinc (at concentrations of 100 μM or higher) inhibited the activation of DEVD-caspase induced by 4 mM butyrate as well as basal DEVD-caspase activity in the control cells. A lower physiological concentration of zinc (25 μM or higher) was effective when zinc ionophore was also present, since this ionophore facilitates zinc uptake across the cell membrane.

8.2.7 Effects of TPEN on p21 and its cleavage

The studies described in chapter 4 have suggested that the cell cycle regulator p21 is a specific substrate of caspase-3 in cells induced to apoptose by butyrate. In order to determine whether p21 was also cleaved in TPEN-treated cells, LIM1215 cells were treated with 25 μM TPEN for varying periods of time and then lysates assayed by Western blotting with a monoclonal anti-p21 antibody. This antibody also detects the larger p15 cleavage product of p21, formed by caspase-3-dependent cleavage of p21 at D_{112}. Decrease in p21 and appearance of p15 first occured 1 h after addition of TPEN (Fig 8.16 A&B). By 2 h, most of the p21 had been cleaved and there was a major band corresponding to p15. By 4h, neither p21 nor p15 were detectable, suggesting further degradation of p15.

Since 1 mM butyrate is synergistic with TPEN, priming with 1mM butyrate may promote the cleavage. To test this, LIM1215 cells were primed with 1 mM butyrate for 18 h, and then the cells further incubated with 25 μM TPEN for varying periods of time, and

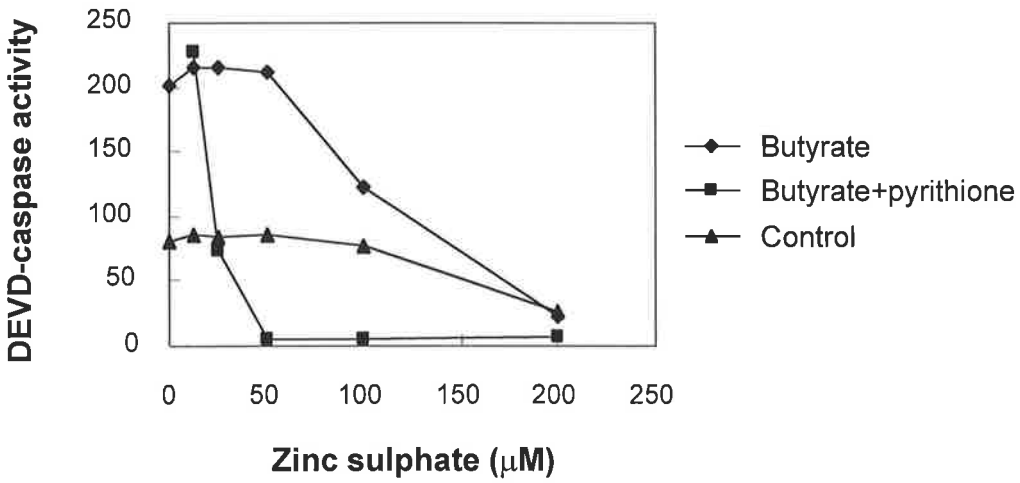


Figure 8.15 Inhibition of the activation of butyrate-induced DEVD-caspase by zinc and pyrithione in LIM 1215 cells. $8x10^5$ cells were seeded into wells. Two days after, the cells were pretreated with $ZnSO_4$ (12.5-200 μM) for 1 h before addition of 4 mM butyrate (filled rhombuses) or control solvent (filled triangles) for a further 24 h. A third set of wells (filled squares) contained 1 μM sodium pyrithione as a zinc ionophore, in addition to $ZnSO_4$, to promote cellular uptake of zinc. This graph shows that zinc, at concentrations of 100 μM or higher, inhibits the activation of DEVD-caspase induced by 4 mM butyrate as well as basal DEVD-caspase activity in the control cells. A lower concentration of zinc (25 μM or higher) was effective when zinc ionophore was also present. Data are means of duplicates.

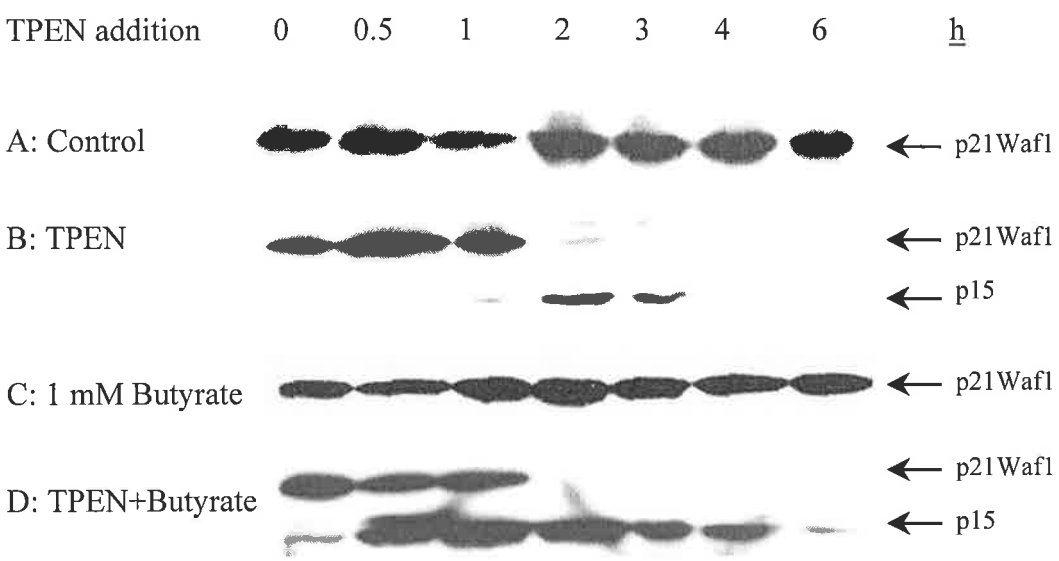


Figure 8.16 Western blot showing the cleavage of p21 in TPEN-treated LIM 1215 cells. $1.5x10^6$ cells were seeded into 25 cm^2 flasks. Two days after, cells were pretreated with 1mM butyrate for 18 h and then were treated with 25 μM TPEN. At the times indicated, the cells were collected for Western blotting. This figure shows that the loss of p21 was associated with appearance of p15, followed later by disappearance of both bands. This process occurred more rapidly in cells primed with butyrate before addition of TPEN.

A: Untreated LIM 1215 cells.

B: TPEN-treated LIM 1215 cells.

C: 1mM butyrate-treated LIM1215 cells.

D: LIM1215 cells pre-treated with 1mM butyrate before addition of TPEN. Note that at time 0 there was a small amount of p15, suggesting that some cleavage of p21 had occurred during priming with 1mM butyrate. Also note that p15 band persisted longer than in cells treated with TPEN alone (B). The reason for this is not clear.

lysates were assayed by Western blotting (Fig 8.16 C&D). Priming with 1mM butyrate resulted in a more rapid onset of p21 cleavage, occurring as early as 30 min after addition of TPEN (Fig 8.16 D).

8.3 DISCUSSION

The aim of the experiments in this chapter was to investigate the involvement of zinc in the apoptosis of LIM1215 cells. Previous studies have shown that a) intracellular zinc levels were higher in colon cancer tissue than the adjacent mucosa, and b) zinc deficiency in vivo is associated with enhanced apoptotic cell death in epithelial tissues and may play an important role in the damage to the gastrointestinal lining and associated diarrhoea. The experiments in this chapter lead to four major conclusions: 1) apoptosis occurs in zinc-depleted CRC cells and is accompanied by activation of caspase-3 and caspase-6; 2) there is only a short lag period (2 h) between depletion of intracellular zinc and onset of caspase-3 activation; 3) zinc depletion enhances butyrate-induced apoptosis while zinc supplementation suppresses it; and, 4) activation of caspase-3 by zinc depletion is rapidly followed by loss of $p21^{Waf1/Cip1}$, first to a 15KDa fragment which is then further degraded.

The studies with Zinquin confirmed that LIM1215 cells had appreciable levels of labile zinc in their cytoplasm and this zinc was rapidly chelated by TPEN. It is not yet possible to calculate the actual concentration of zinc from the zinquin fluorescence data. This pool of zinc is clearly important in the regulation of apoptosis. Apoptosis was confirmed in zinc-depleted LIM1215 cells by four independent criteria: morphological criteria, flow cytometric analysis, DNA fragmentation and DEVD-caspase activation. Apoptosis was also confirmed in zinc-depleted SW480 cells. The findings in TPEN-

treated cells of a time- and concentration-dependent increase in DEVD-caspase activity, loss of the 32 KDa zymogen form of caspase-3, and partial suppression of DNA fragmentation by caspase peptide inhibitors (including a specific inhibitor of caspase-3, zDEVD-fmk), argue for a major role of caspase-3 in the TPEN-induced apoptosis. This conclusion is strengthened by the recent study of TPEN-induced cell death in human renal cell carcinoma cell lines, where mutant cell lines lacking caspase-3, were resistant to TPEN-induced apoptosis (Kolenko *et al.*, 1999). Since these resistant cell lines also had variable decreases in caspase-7, caspase-8 and caspase-10, it cannot be ruled out that loss of one of these upstream caspases may also have been important.

Caspase-6, which is the caspase responsible for cleavage of nuclear lamins (Takahashi *et al.*, 1996), was also activated in TPEN-treated CRC cells in this study and this caspase may be responsible for the subsequent nuclear fragmentation in zinc-depleted cells. It is interesting that the suppression of TPEN-induced DNA fragmentation by the caspase-3 peptide inhibitor zDEVD-fmk was only partial, despite complete suppression of DEVD-caspase activity. That other caspases may substitute for caspase-3 is unlikely since the broad spectrum caspase inhibitor zVAD-fmk was also only partially able to suppress DNA fragmentation. Some of the resulting DNA fragmentation is likely to be via a caspase-independent mechanism. The observations that TPEN lowers intracellular zinc under the conditions used in this study and that zinc supplementation prevents the effects of TPEN on apoptosis of these cells, suggests that depletion of zinc is responsible for most, if not all, of the actions of TPEN on these cells.

The mechanism by which intracellular zinc depletion triggers caspase-3 activation in CRC cells remains unclear. In view of the short lag period (1-2 h) between addition of

TPEN and onset of caspase-3 activation, zinc must influence a step relatively close to that of caspase-3 activation. Caspase-3 activation involves the proteolytic processing of a 32 KDa zymogen by other caspases; both caspase-9 (Slee *et al.*, 1999) and caspase-6 (Liu *et al.*, 1996) have been implicated in this processing. It is of interest, that both of these caspases are very sensitive to inhibition by zinc (Wolf and Eastman, 1999; Takahashi *et al.*, 1996; Stennicke and Salvesen, 1997). TPEN might therefore act by stripping zinc from either or both of these caspases, enhancing their activity. It was not possible to show any significant activation of caspase-6 prior to activation of caspase-3. Caspase-6 was activated in TPEN-treated cells, but only at time-points later than that of caspase-3 activation. It is possible that only small amounts of caspase-6 are required for processing of caspase-3 and that these levels were too low to be detected by the fluorogenic substrate assay. Effects of zinc depletion on caspase-9 activity now need to be determined. Alternatively, zinc may influence the levels of auxiliary factors in the activation of caspase-3. Candidates include members of the Bcl-2 family of proteins which suppress caspase-3 activation and may be up-regulated by zinc supplementation (Fukamachi *et al.*, 1998). Another candidate may be nitric oxide, since a nitric oxide synthase inhibitor protected mice from zinc deficiency-induced apoptosis and damage in the gastrointestinal epithelium (Cui *et al.*, 1999).

The third conclusion of this study was that butyrate-induced apoptosis was strongly influenced by the zinc status of the cells. Increases of zinc suppressed the apoptosis while decreases enhanced it. This may be clinically relevant. In view of the finding by Song et al (Song *et al.*, 1993) that colon cancers may have increases in the level of zinc, this metal could render them less susceptible to the protective effects of butyrate

in vivo. Secondly, physiological factors which act to deplete intracellular zinc (eg phytic acid in high fibre diets), may facilitate the protective effects of butyrate in vivo (see chapter 9).

The fourth major conclusion of this study was that activation of caspase-3 in zinc-depleted CRC cells, was rapidly followed by loss of the cell-cycle regulator p21. This loss proceeded in two steps: initially, there was cleavage of p21 to a 15KDa derivative, previously shown in chapter 4 and by others (Donato and Perez, 1998; Levkau *et al.*, 1998; Gervais *et al.*, 1998) to arise by caspase-3-dependent cleavage of p21 at D_{112}. Following the initial cleavage of p21, there was complete loss in zinc-depleted cells. This occurred in spite of little changes in total protein content of the cells, indicating that the loss of p21 is specific. Since the turnover of p21 is regulated by the proteasome complex of proteases (Blagosklonny *et al.*, 1996), the initial cleavage to p15 may expose sites that facilitate ubiquitination and subsequent targeting to the proteasome. Loss of p21 may result in premature entry of the cells into S-phase and apoptotic cell death, as occurs in some other forms of apoptosis (King and Cidlowski, 1998). Experiments to test the effect of zinc depletion on apoptosis in murine cells or in cells transfected with a mutant form of p21 that has an alanine instead of aspartate at position 112 and which will, therefore, be resistant to caspase-dependent cleavage, should prove informative.

To what extent in vivo zinc deficiency is associated with enhanced rates of caspase activation, p21 degradation and apoptosis in normal and malignant colorectal tissues needs to be determined. This will best be answered in the context of experimental zinc deficiency in animal models of colorectal tumorigenesis.

Some of the results in this chapter are published in (Chai *et al.*, 2000).

CHAPTER 9

THE EFFECTS OF PHYTIC ACID ON APOPTOSIS OF COLORECTAL CANCER CELLS

9.1 INTRODUCTION

Dietary fibre comprises a group of non-starch polysaccharides such as cellulose, hemicellulose, and pectin and non-carbohydrate substances such as phytic acid (inositol hexaphosphate, IP_6). The protective effect of these agents on colorectal cancer depends on the nature and source of fibre in the diet. As discussed already, the short chain fatty acid butyrate, a colonic fermentation product of dietary polysaccharides, partly mediates their protective action and may protect via modulation of expression of colonic epithelial genes involved in cell-cycle arrest, differentiation and apoptotic cell death. As a component of dietary fibre, phytic acid has been studied extensively for its chemopreventive properties against colon carcinogenesis in laboratory animal models. Phytic acid reduced the number of colon tumours induced by the carcinogen azoxymethane as well as the size of tumours (Shamsuddin *et al.*, 1988). Also, dietary phytic acid reduced the incidence of colonic aberrant crypt foci in rats (Jenab and Thompson, 1998; Challa *et al.*, 1997). Furthermore, it was shown that inhibition of colon cancer was dose-dependent (Ullah and Shamsuddin, 1990). Oral administration of phytic acid inhibited colon carcinogenesis in rodents not only in the initiation stages but in post-initiation stages as well (Jenab and Thompson, 1998). Although phytic acid can normalise the increased mitotic rate induced by carcinogen, it does not affect the mitotic rate in normal animals (Shamsuddin *et al.*, 1988; Shamsuddin and Ullah, 1989; Shamsuddin *et al.*, 1989; Ullah and Shamsuddin, 1990). The study by Jenab and Thompson (Jenab and Thompson, 2000) concluded that part of the protective action of wheat bran is due to phytic acid and that exogenous phytic acid when added to low fibre diet can increase both apoptosis and differentiation in colonocytes in vivo.

Also, in vitro experiments showed that treatment with phytic acid resulted in the inhibition of proliferation and induction of differentiation in colorectal (Yang and Shamsuddin, 1995), mammary (Shamsuddin *et al.*, 1996) and prostate cancer cell lines (Shamsuddin and Yang, 1995).

Although it is known that phytic acid acts as an anti-oxidant to reduce the rate of cell proliferation and to augment the immune response (Reddy, 1999), the anti-tumour mechanism is not clear. However, dietary phytic acid is highly negative charged because of its phosphate groups. Therefore, phytic acid can chelate zinc, and reduce its absorption, resulting in zinc deficiency in vivo (Lonnerdal *et al.*, 1988; Navert *et al.*, 1985; Pecoud *et al.*, 1975). It has been shown that intact phytic acid can be rapidly transported into cells in vivo (Sakamoto *et al.*, 1993) and in vitro (Vucenik and Shamsuddin, 1994). Once inside the cell phytic acid may chelate intracellular zinc, thereby inhibiting the function of metalloproteins, such as transcription factors that are important in gene regulation (O'Halloran, 1993) or proteins involved in regulation of apoptosis. Evidence from in vitro studies described in chapter 8 indicates that apoptosis can result directly from a decline in intracellular zinc induced by culture of cells in the presence of membrane-permeant zinc chelators, such as TPEN.

Of interest, phytic acid has been shown to mimic butyrate in increasing the differentiation of a colon cancer cell line (Sakamoto *et al.*, 1993). The effects of phytic acid on apoptosis of colon cancer cell lines have not yet been examined. In this study, it was therefore tested (1) whether phytic acid can induce apoptosis in CRC cells and if so, (2) whether phytic acid is synergistic with butyrate in its ability to induce apoptosis and (3) whether phytic acid acts by depleting intracellular zinc.

9.2 RESULTS

9.2.1 Phytic acid induces caspase-3 activation and DNA fragmentation and apoptosis

To determine the effects of phytic acid on apoptosis of colorectal cancer cells, LIM 1215 cells were seeded into six-well plates and treated with various concentrations (0-10 mM) of phytic acid for 24 h. Apoptosis was assessed by morphology, DNA fragmentation and DEVD-caspase activation. Examination of phytic acid-treated cells by bright field microscopy showed typical morphological features of apoptosis including the formation of apoptotic bodies, membrane blebbing and reduction in volume (Fig 9.1B), compared with the normal morphology of these cells before treatment with phytic acid (Fig 9.1A). Labelling with Hoechst dye 33342 revealed segmentation of the chromatin into discrete masses in the phytic acid-treated cells (Fig 9.1D), compared with the uniform mass of chromatin in untreated cells (Fig 9.1C). Apoptosis was also confirmed by increase of DNA fragmentation in cell lysates (Fig 9.2, filled squares). DNA fragmentation began to increase between 5 and 10 mM phytic acid. As for DEVD-caspase activity, there was a small, significant ($p \leq 0.005$) increase with 5 mM phytic acid, followed by larger increases ($p \leq 0.005$) at 10 mM (Fig 9.2, filled diamonds).

To confirm these findings in another colorectal cancer cell line, SW480 cells were used. Both DNA fragmentation and DEVD-caspase activity began to increase beyond 5 mM phytic acid, up to 10-20 mM phytic acid (Fig 9.3). The level of DEVD-caspase activity reached in SW480 cells was similar to that in LIM1215 cells. The level of DNA fragment was a little less.

The kinetics of these increases was studied using 7 mM phytic acid. DEVD-caspase activity increased slowly within 12 h after addition of phytic acid in LIM1215

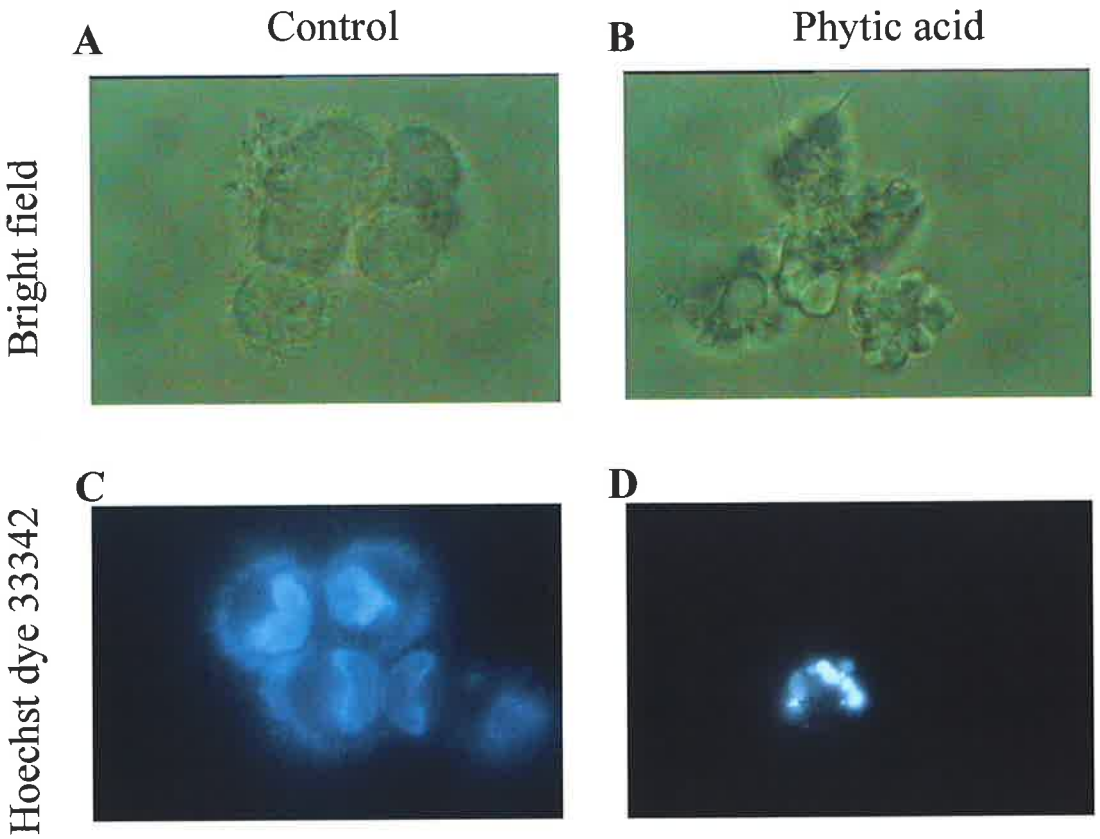


Figure 9.1 Apoptotic morphology of phytic acid-treated LIM1215 cells. $6x10^5$ LIM1215 cells were seeded into wells and, after 2 days, treated for 24 h with 7 mM phytic acid. Cells were washed with HBSS (3X) before addition of Hoechst dye 33342 (final concentration 110μg/ml) for a further 5min to stain the chromatin. Cells were examined under bright field illumination or UV epifluorescence. Images were captured and stored using a video camera and image analysis software.

A & B: Bright field illumination of typical cells are shown for control (A) and phytic acid-treated cells (B). Initial magnification (2500x). Note the blebbing of the cells and formation of apoptotic bodies in B.

C& D: UV epifluorescence of typical cells are shown for control (C) and phytic acid-treated cells (D). Initial magnification (2500x). Note the fragmentation of chromatin in D.

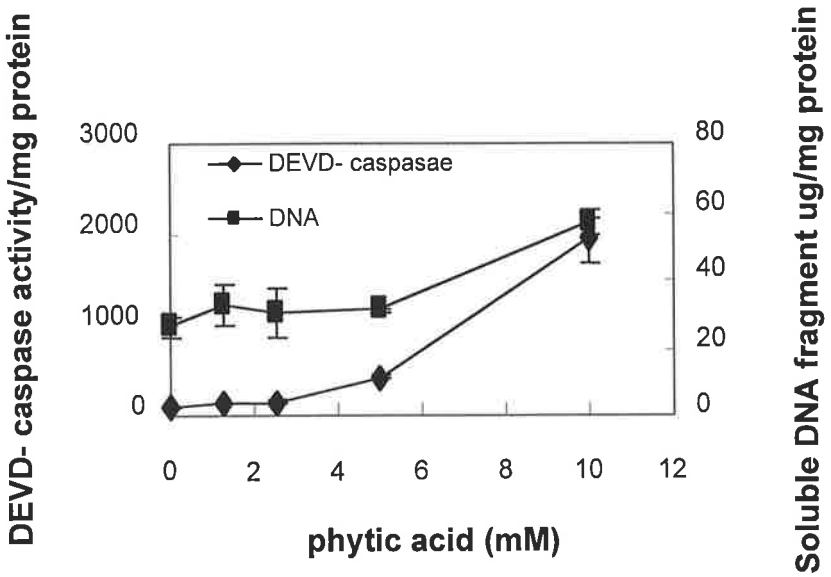


Figure 9.2 DEVD-caspase activity and DNA fragmentation induced by phytic acid in LIM 1215 cells. $6x10^5$ cells were seeded into 6-well plates. Two days after, the cells were treated with varying concentrations of phytic acid (0-10 mM) for 24 h. Thereafter, cells were harvested for DEVD-caspase activity (filled diamonds) and DNA fragmentation (filled squares). This graph shows that DEVD-caspase activity increased at concentrations of phytic acid beyond 2.5 mM, while DNA fragmentation increased beyond 5 mM. Data are the means of triplicates; error bars indicate the standard deviations.

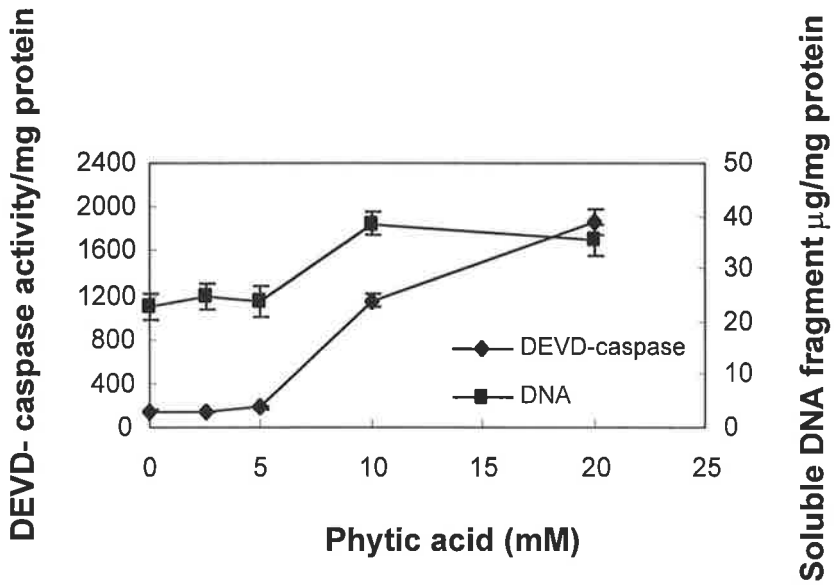


Figure 9.3 DEVD-caspase activity and DNA fragmentation induced by phytic acid in SW480 cells. $6x10^5$ cells were seeded into 6-well plates. Two days after, the cells were treated with varying concentrations of phytic acid (0-20 mM) for 24 h. Thereafter, cells were harvested for DEVD-caspase activity (filled diamonds) and DNA fragmentation (filled squares). This graph shows that both DEVD-caspase activity and DNA fragmentation increased markedly at concentrations of phytic acid between 5 and 10 mM. Data are the means of triplicates; error bars indicate the standard deviations.

cells (Fig 9.4). After 12 h, there were sharp rises in DEVD-caspase activity, increasing linearly up to 24 h; thereafter, DEVD-caspase activity began to decrease. Similar results for DNA fragmentation were found except that while DNA fragmentation increased beyond 24 h, DEVD-caspase activity decreased (Fig 9.5). The likely reason for this is that with increasing time there are increasing apoptosis and DNA fragmentation; however, caspase-3 activity peaked at 24 h and decreased thereafter.

9.2.2 Interaction of phytic acid and butyrate on LIM1215 cells

LIM1215 cells were treated with varying concentrations of butyrate (0-4 mM) in the presence or absence of 5 mM phytic acid. Fig 9.6 shows that a combination of 5 mM phytic acid with 1, 2 or 4 mM butyrate resulted in changes of DEVD-caspase activity, that were 23% lower (not significant), 11% higher (not significant), or 15% lower ($p \leq 0.05$) respectively, than the additive effects of the treatments with the reagents alone. None of these data are significantly higher than the additive effects alone, suggesting a lack of synergy between the two reagents. The data may even suggest that butyrate and phytic acid interact in a negative sense. However, it is not clear how this might occur. Possibly a combination of the two results in some necrosis.

9.2.3 Role of zinc depletion in the induction of apoptosis by phytic acid

Since depletion of intracellular zinc by TPEN induces apoptosis, and phytic acid is a known zinc chelator, it is possible that phytic acid also causes apoptosis by making the cells zinc deficient. To test this, the following experiments were performed.

Firstly, I tested whether the concentrations of phytic acid had induced apoptosis significantly chelated environmental zinc. To do this, the UV-excitable zinc-specific

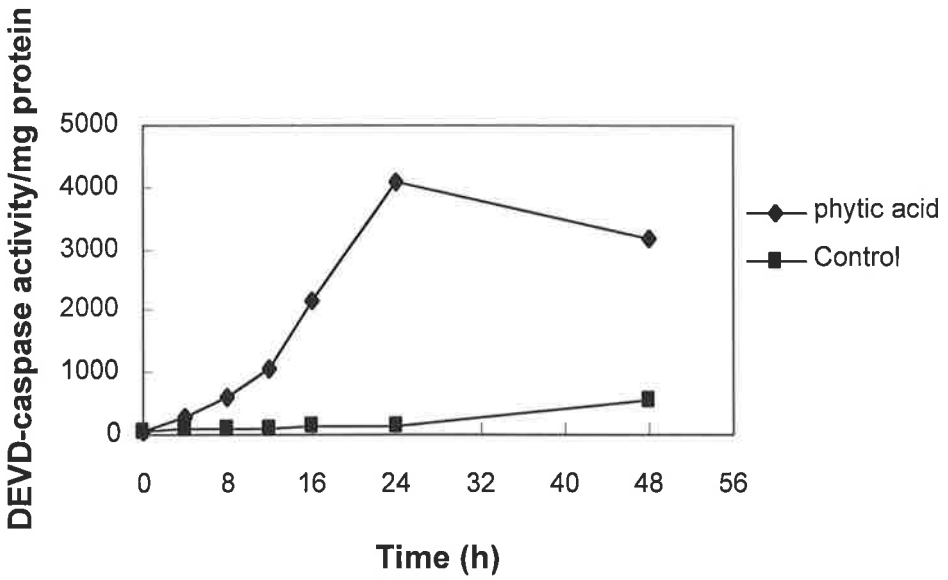


Figure 9.4 Time course of DEVD-caspase activity induced by phytic acid in LIM1215 cells. $6x10^5$ cells were seeded into 6-well plates. Two days after, the cells were treated with 7mM phytic acid for varying periods of time (up to 48 h). Thereafter, cells were harvested for DEVD-caspase activity. This graph shows that DEVD-caspase activity increased slowly in the first 12 h in phytic acid-treated cells (filled diamonds) and then more rapidly over the next 12 h. There was no further increase up to 48 h. Control cells (filled squares) showed only a slight increase after 48 h. Data are the means of triplicates; error bars indicate the standard deviations (obscured by the symbols).

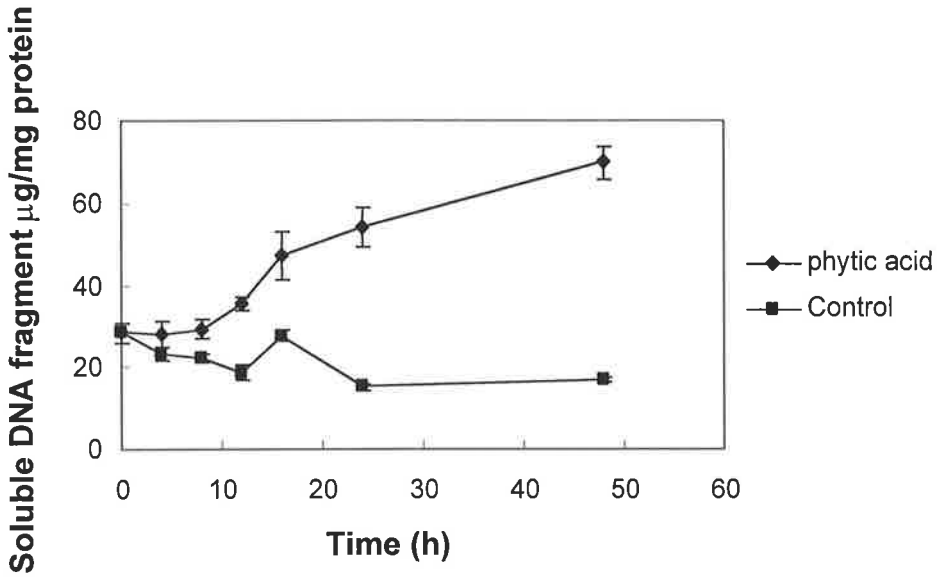


Figure 9.5 Time course of DNA fragmentation induced by phytic acid in LIM1215 cells. $6x10^5$ cells were seeded into 6-well plates. Two days after, the cells were treated with 7mM phytic acid for varying periods of time (up to 48 h). Thereafter, cells were harvested for DNA fragmentation. This graph shows that DNA fragmentation increased after a lag period of 10 h in phytic acid-treated cells (filled diamonds) and continued to increase up to 48 h. Control cells (filled squares) showed no increase up to 48 h. Data are the means of triplicates; error bars indicate the standard deviations.

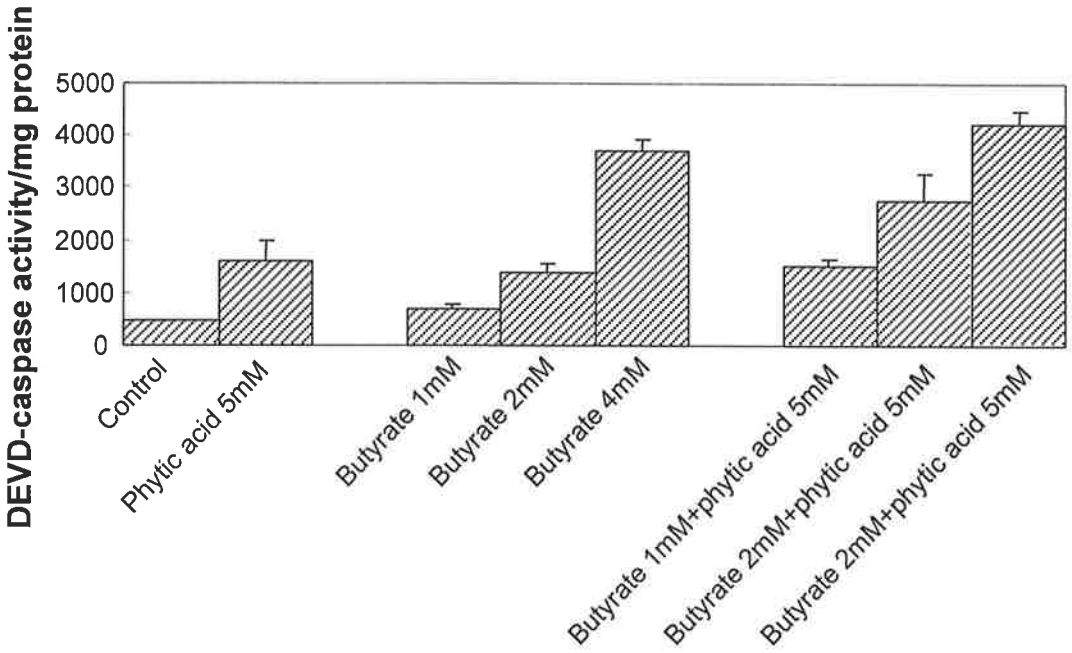


Figure 9.6 Lack of synergy between butyrate and phytic acid on activation of DEVD-caspase in LIM 1215 cells. $6x10^5$ cells were seeded into 6-well plates. Two days after, the cells were treated with 0, 1, 2 or 4 mM butyrate for 24 h in the presence or absence of 5 mM phytic acid. Thereafter, cells were harvested for DEVD-caspase activity. This figure shows that combination of 5 mM phytic acid with 1, 2 or 4 mM butyrate resulted in increases of DEVD-caspase activity, that were 23% lower, 11% higher, or 15% lower respectively, than the additive effects of the treatments with the reagents alone. This indicates a lack of synergy between the two reagents and a possible negative interaction between them. Data are the means of triplicates; error bars indicate the standard deviations.

fluorophore Zinquin (1 μM) was incubated with 2 μM $ZnSO_4$ in the presence or absence of varying concentrations of phytic acid (0-20 mM). As a positive control, TPEN (0-25 μM) was used. Zinquin fluorescence was then measured in a spectrofluorometer (excitation wavelength of 365 nm and emission wavelength of 485 nm). A decrease of fluorescence indicates a decrease in the zinc concentration. Fig 9.7 shows that there was no decrease in Zinquin fluorescence with phytic acid (0-20 mM); actually there was an increase in Zinquin fluorescence. The reason for this is not clear. In contrast, TPEN at ≥ 3.13 μM significantly reduced the Zinquin fluorescence, with complete quenching of fluorescence at ≥ 6.25 μM.

Secondly, I tested whether the concentrations of phytic acid used to induce apoptosis significantly chelated intracellular zinc in LIM1215 cells. LIM1215 cells were treated with phytic acid (5 mM) or no addition for 2 h. Cells were then washed and incubated with 25 μM Zinquin for further 30 min. Images of the cells under UV epifluorescence were captured and stored using a video camera and the Video Pro image analysis software package. Fluorescence was quantified in 200 cells from 10 different fields for each treatment. Fig 9.8 shows a typical field for control (A) and phytic acid-treated cells (B). Zinquin fluorescence images of the cells are shown in (C) and (D). Note the cytoplasmic fluorescence and the lack of any decrease of Zinquin fluorescence after treating with phytic acid, indicating no effects on labile intracellular pools of zinc. This is in contrast to the pronounced quenching of fluorescence by TPEN (see chapter 8). Mean fluorescence intensity of control cells was 13.96±7.72 pixels (n=242) and for phytic acid-treated cells was 14.62±6.57 pixels (n= 188, $p>0.05$).

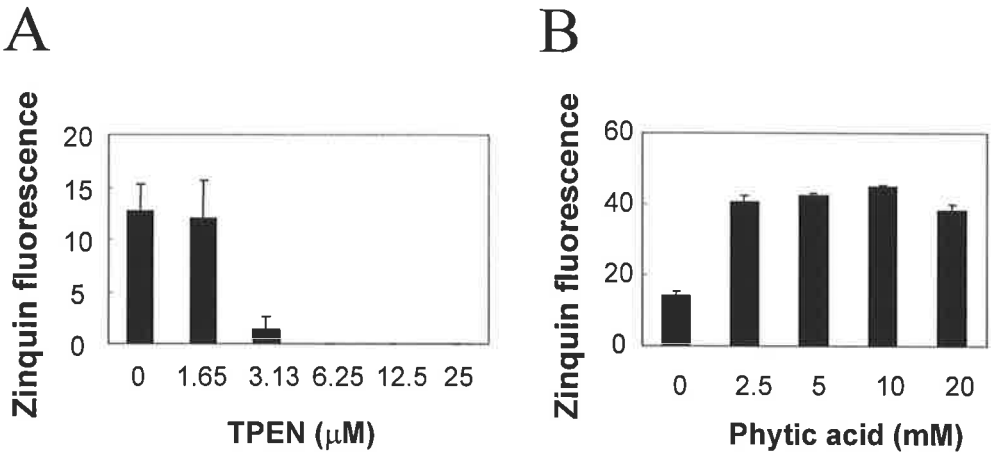


Figure 9.7 In vitro zinc chelation by TPEN but not by phytic acid. In disposable cuvettes, 0.5 ml of HBSS containing 1mg/ml BSA and 2 μM zinc sulphate, was mixed with 0.25 ml of in HBSS containing TPEN (0-25 μM final concentration in HBSS) or phytic acid (0-20 mM final concentration). After 10min at room temperature, 0.25 ml of HBSS containing 1mg/ml BSA and 1 μM Zinquin was added. Fluorescence was read in a spectrofluorometer at excitation wavelength of 365 nm and emission wavelength of 485 nm with 10nm slit widths. Fluorescence of the blank was subtracted to give specific Zinquin fluorescence. **A**: This figure shows that TPEN (≥ 3.13 μM) chelated zinc as determined by the decrease in Zinquin fluorescence. **B**: Phytic acid did not chelate zinc at the concentrations tested. In fact, fluorescence increased. Data are the means of triplicates; error bars indicate the standard deviations.

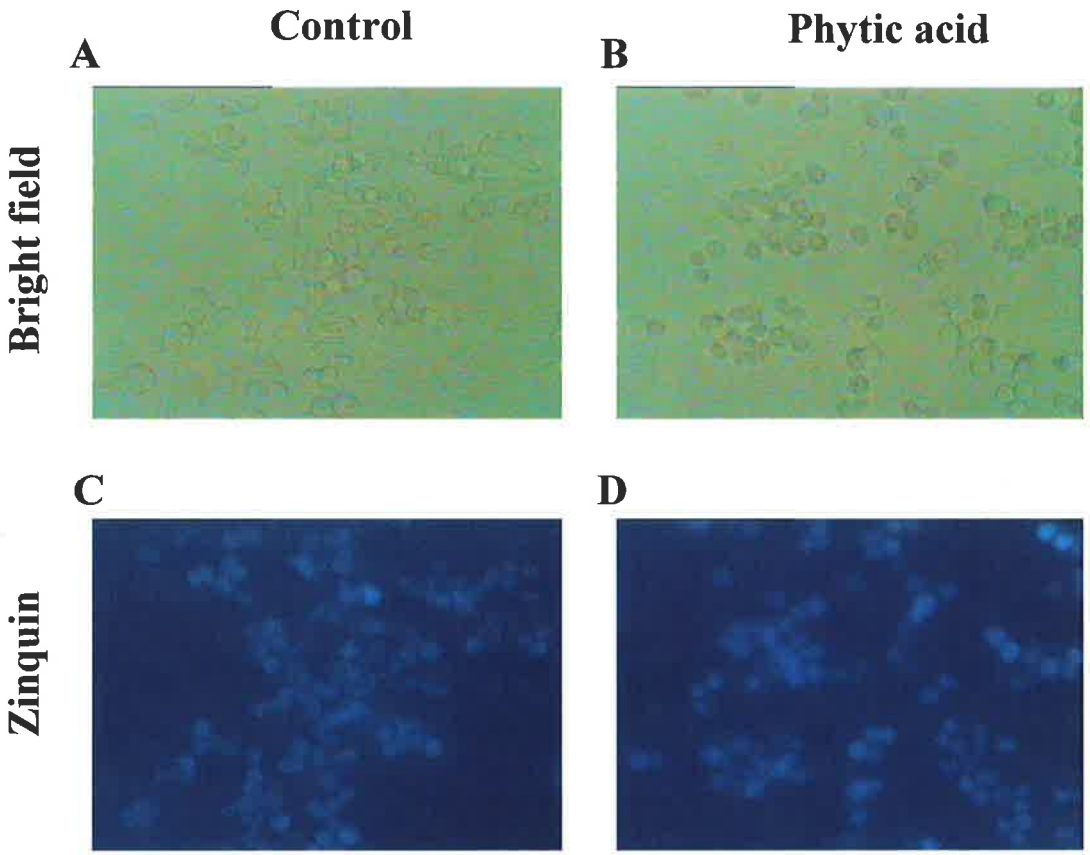


Figure 9.8 Zinc depletion by phytic acid in LIM 1215 cells. Coverslip monolayers of LIM1215 cells were treated for 2 h in the presence of 5mM phytic acid. Cells were washed with HBSS (3X) before addition of 25μM Zinquin for a further 30 min. Coverslips were inverted on to microscope slides and examined under UV epifluorescence. Images were captured and stored using a video camera and image analysis software. Fluorescence intensity was quantified in 200 cells from ten different fields. Mean fluorescence intensity was calculated.

A & B: Bright field images of typical fields for control (A) and phytic acid-treated cells (B). Initial magnification(500x)

C& D: Zinquin fluorescence images of typical fields for control (C) and phytic acid-treated cells (D). Initial magnification(500x)

To further confirm that zinc is not involved in phytic acid-induced apoptosis, an experiment of zinc supplementation was done to test whether zinc blocks phytic acid-induced apoptosis. LIM1215 cells were treated with phytic acid in the presence or absence of zinc sulphate. The induction of DEVD-caspase activity by 5 mM phytic acid was not prevented, when cells also received exogenous $ZnSO_4$ (25 μM) (Fig 9.9). Thus, after 24 h treatment of LIM1215 cells with phytic acid, there was a 17.8-fold increase in DEVD-caspase activity over the control (no phytic acid). When cells were treated with phytic acid in the presence of 25 μM $ZnSO_4$, this increase was 18.5-fold. These values are not significantly different. This is in contrast to the strong inhibition of TPEN-induced DEVD-caspase activation by 25 μM $ZnSO_4$ (see chapter 8). These studies with exogenous zinc do not support the hypothesis that phytic acid is acting by chelating zinc.

9.3 DISCUSSION

The objective of these experiments was to test the hypothesis that phytic acid, a component of dietary fibre, induces apoptosis of CRC cells and, if so, to determine whether this results from depletion of cellular zinc. A lot of evidence has accumulated to show that phytic acid can inhibit proliferation and induce differentiation of tumour cells in vitro or in vivo. The molecular mechanism is not clear. Increases in apoptosis in vivo have been shown in aberrant crypts of rats, where phytic acid is given either in food or as a supplement (Jenab and Thompson, 2000). Here, I have shown for the first time, that 1) phytic acid kills colorectal cancer cells in vitro by apoptotic death, 2) there was no synergy with butyrate and 3) apoptosis was not mediated by depletion of intracellular zinc.

Treatment of colorectal cancer LIM1215 cells with phytic acid for 24 h resulted in typical morphological feature of apoptosis. Paralleling the morphological changes, were

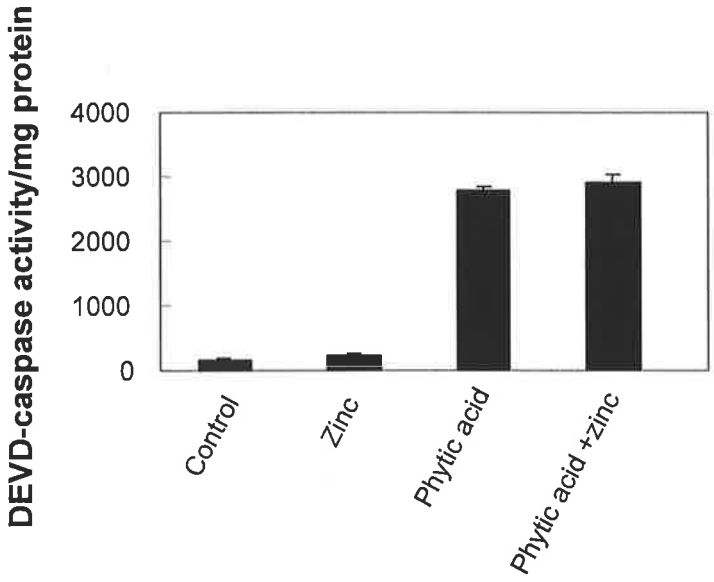


Figure 9.9 Zinc supplementation does not block phytic acid-induced DEVD-caspase activity in LIM 1215 cells. $8x10^5$ cells were seeded into 25cm^2 flasks. Two days after, the cells were pretreated with $ZnSO_4$ (25 μM) for 1 h before addition of 5mM phytic acid for a further 24 h. This graph shows that zinc does not inhibit the activation of DEVD-caspase induced by phytic acid. Data are the means of triplicates; error bars indicate the standard deviations.

activation of DEVD-caspase and DNA fragmentation. The experiment showed that induction of DEVD-caspase was concentration- and time-dependent. All these results support that phytic acid-induced cell death is apoptotic death. This finding is consistent with the observation in vivo (Jenab and Thompson, 2000). Phytic acid-induced apoptosis was also confirmed in SW480 cells. These results suggest that the anti-carcinogenic action of phytic acid may be via apoptosis of tumour cells, as well as by inhibition of proliferation and induction of differentiation as previously reported (Yang and Shamsuddin, 1995; Shamsuddin and Yang, 1995; Shamsuddin *et al.*, 1996). The extracellular concentrations of phytic acid that were effective in inducing apoptosis of LIM1215 and SW480 cells were in the range of 5-10 mM. Because phytic acid contains a high negative charge due to its six phosphate groups, it may be poorly incorporated into cells. However, work from Shamsuddin's laboratory (Vucenik and Shamsuddin, 1994), has shown that phytic acid is rapidly taken up by malignant cells in vitro. It is not known whether the intracellular concentrations of phytic acid achieved were physiological, but cells have been reported to contain up to 1 mM intracellular phytic acid (Shamsuddin *et al.*, 1997).

Both butyrate and phytic acid are present in normal colon, as a result of consumption of fibre. The lack of synergy between butyrate and phytic acid in inducing apoptosis, is interesting and suggests that these two agents may act by a common pathway, although this needs further investigation. Of interest, Saied and Shamsuddin (Saied and Shamsuddin, 1998) have recently reported that phytic acid up-regulates the expression of the tumor suppressor gene p53 and the p21 gene. Hence, a common action of butyrate and phytic acid may be at the level of up-regulation of these proteins, although whether this

contributes to the apoptosis is unclear. Whether either agent is more important than the other in protection against colorectal cancer and whether they act synergistically or additively in animal models of colorectal cancer remain to be determined.

Phytic acid is also known to chelate dietary zinc in the intestine, thereby preventing the absorption of zinc (Lonnerdal *et al.*, 1988; O'Dell, 1969; Sandstrom *et al.*, 1983; Vohra and Kratzer, 1966). One possible mechanism for action of phytic acid is by depletion of intracellular zinc in LIM1215 cells, resulting in the activation of DEVD-caspase and downstream events of apoptosis. The lack of synergy between butyrate and phytic acid in induction of apoptosis of LIM1215 cells is in contrast to that between butyrate and another zinc chelater TPEN (see chapter 8). This suggests phytic acid-induced apoptosis does not involve depletion of intracellular zinc like TPEN. In support of this hypothesis, phytic acid failed to chelate extracellular zinc and intracellular zinc at the concentrations used to induce apoptosis, while as a positive control, TPEN, under the same conditions, chelated both environmental and intracellular zinc. It may be that phytic acid has a much lower affinity for zinc than does Zinquin. It was not possible to obtain a published dissociation constant of phytic acid for zinc. The dissociation constant of Zinquin for zinc is in the nM range (Zalewski *et al.*, 1993). A third piece of evidence that phytic acid does not act via depletion of zinc is that addition of zinc to the medium did not block phytic acid-induced DEVD-caspase activation, although it strongly inhibited TPEN-induced DEVD-caspase activation.

Taking all of this evidence together, it is likely that phytic acid induces apoptosis by a mechanism independent of its effect on intracellular zinc. Phytic acid also chelates iron ; Vohra *et al.*, 1965) and calcium (Kaufman and Kleinberg, 1971; Vohra *et al.*, 1965).

Whether chelation of these metals is involved in the apoptosis is not clear. The mechanism may also involve inhibition of phosphatidylinositol-3' kinase. For example, it has been reported that phytic acid can inhibit EGF- or TPA-induced cell transformation as well as the signal transduction cascade leading to activation of the Erks family of tyrosine kinases and the downstream activation of transcription factor AP-1 in vivo and in vitro, by suppressing activation of phosphatidylinositol-3' kinase (Huang *et al.*, 1997). Phosphatidylinositol-3-kinase has been reported to be an anti-apoptotic factor in primary human epithelial cells (Gire *et al.*, 2000).

In conclusion, phytic acid does induce DEVD-caspase activation, DNA fragmentation and morphological changes of apoptosis, like the other dietary fibre-associated factor butyrate. The two agents did not act synergistically and phytic acid did not induce apoptosis by depletion of cellular zinc.

CHAPTER 10

FINAL DISCUSSION AND CONCLUSIONS

The main aims of this thesis were, 1) to study the mechanism by which butyrate (the main fermentation product of dietary fibre), induces apoptosis of colorectal cancer cells, and 2) to determine the effect of butyrate in combination with NSAIDs (chemopreventive agents) and other agents on colorectal cancer cells. The experiments mainly focussed on the following issues: 1) The relationship of butyrate-induced growth arrest to apoptosis in colorectal cancer cells; 2) the relationship of $p21^{Waf1/Cip1}$ (p21) and retinoblastoma protein (pRb) to butyrate-induced growth arrest and apoptosis of colorectal cancer cells; 3) the mechanisms of resistance to butyrate; 4) interaction of butyrate and NSAIDs on apoptosis of colorectal cancer cells; 5) the interaction of butyrate and zinc on apoptosis of colorectal cancer cells, especially the effect of depletion of intracellular zinc levels on caspase activation and regulation of p21 in colorectal cancer cells; and 6) the interaction of butyrate and phytic acid on apoptosis of colorectal cancer cells. Four major findings emerged from the study.

Firstly, DEVD-caspase activation is involved in the apoptosis of CRC cells by various dietary and chemopreventive agents (summarised in Fig 10.1). When the experiments in this thesis commenced in 1997, there were only a few papers in the literature describing caspase activity levels in CRC cells, including one from this laboratory. One of the aims of this thesis was to further investigate caspase activation in apoptosis of CRC cells by butyrate and other factors. The DEVD-caspase activity as measured using a fluorogenic substrate in lysates of LIM1215 and SW480 cells, was sensitive, reproducible and time-efficient. There was generally close agreement with DNA fragmentation, morphological changes and flow cytometric method to detect cells with

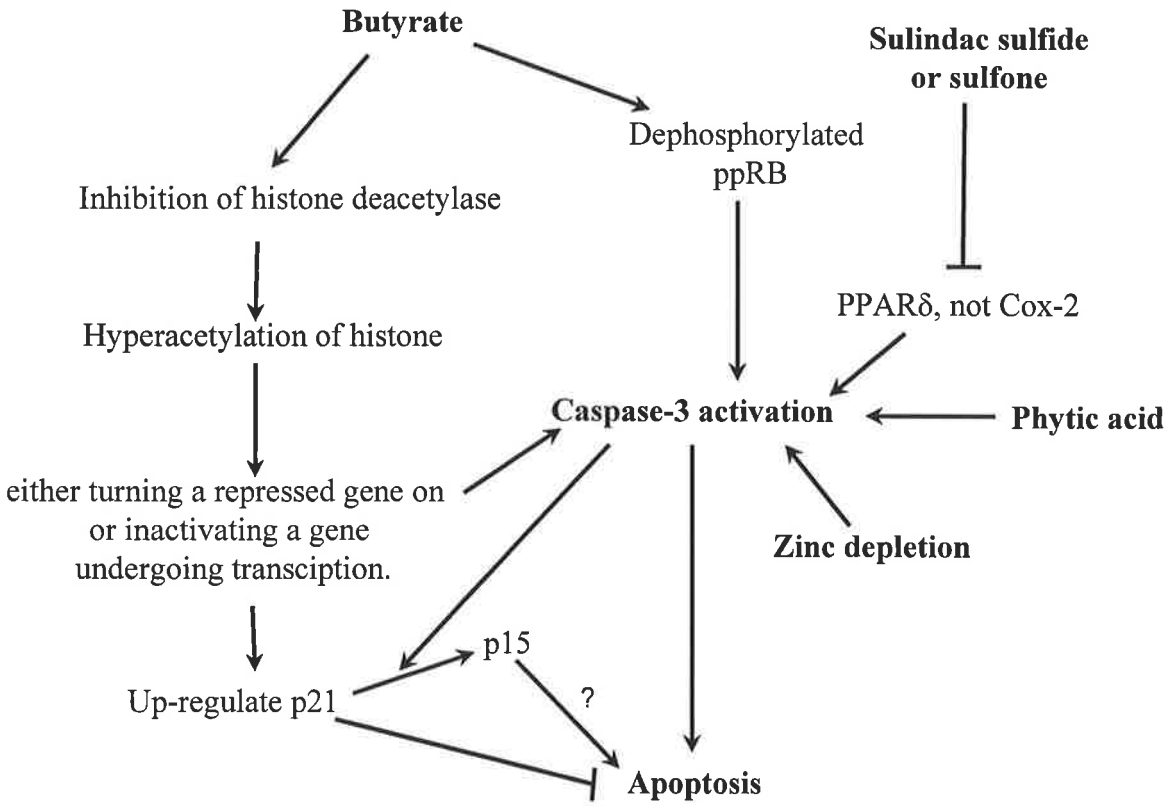


Figure 10.1 Model of butyrate-induced caspase-3 activation and apoptosis in colorectal cancer cells.

"sub-G_1" levels of DNA, all markers of apoptosis. In the transfection studies, there was discrepancy between annexin-V labelling (which indicates the appearance of phosphatidylserine in the outer leaflet of the plasma membrane) and DEVD-caspase activity. For example, butyrate treatment induced substantial DEVD-caspase activity in LIM 1215 cells but there was no increase in Annexin-V labelling, even though the same batch of cells was used. The method for Annexin V labelling was that employed in another laboratory in Adelaide (Department of Orthopaedics and Trauma, Hanson Centre for Cancer Research), where the assay works well. In retrospect, it would have been better to have included a positive control in these studies. Since the transfection experiments were done in the last months of the experimental phase of this study and were time-consuming, it was not possible to repeat them before the submission date of the thesis.

While the caspase enzymatic activities proved to be a useful marker of apoptosis, there were two other discrepancies that are yet to be properly explained. Firstly, in butyrate-treated cells, despite substantial increases in cytosolic DEVD-caspase enzyme levels, there was no decrease in the cpp32 band on Western blots that represents the zymogen form of the enzyme. Since DEVD-caspase activity is also shared by caspases-7 and –2, it could be that it is one of these enzymes rather than caspase-3 which is involved. However, caspase-3 is the most commonly observed caspase to be activated in cells by a variety of stimuli and this explanation seems unlikely. The best way to test this would be by immunoblotting with antibodies specific for the pro-form of these other caspases, however they were not available at the time the experiments were done. Other experiments might test whether other inducers of apoptosis cause loss of cpp32. Interestingly, depletion of Zn with TPEN did lead to a complete loss of cpp32, without

widespread effects on most other proteins in LIM 1215 cells (as observed from the Coomassie-blue stained profiles of the Western blots), suggesting that caspase-3 is turned on in Zn-depleted CRC cells. Since butyrate is known to enhance the transcription of a large number of proteins (via its effects on histone acetylation), it cannot be ruled out that butyrate is increasing the transcription of cpp32. The steady state levels of cpp32 in butyrate-treated CRC cells would then depend upon relative levels of synthesis and proteolytic conversion to caspase-3. This hypothesis is best tested by assaying mRNA levels for cpp32. A probe for cpp32 was not available for my studies.

The other major discrepancy concerns the effect of caspase inhibitors on apoptosis of CRC cells. These peptide inhibitors contain a fluorylmethylketone derivative and are membrane-permeable. DEVD-caspase activity was potently suppressed in the cells even at low concentrations of the inhibitors, but DNA fragmentation and morphological changes of apoptosis were only partially suppressed. This was found not only for butyrate, but also for TPEN, sulindac sulfide and sulindac sulfone in LIM 1215 cells. It cannot simply be that other caspases are involved since similar results were observed with the broad specificity inhibitor zVAD-fmk. Others have reported similar findings in other types of cell and it is now thought likely that there are caspase-independent pathways of apoptosis (Borner and Monney, 1999; Chi *et al.*, 1999; Ha *et al.*, 1998; Jones *et al.*, 1999; Kitanaka and Kuchino, 1999). To what extent the caspase-dependent and-independent pathways interact in butyrate-induced apoptosis is not clear.

The second major finding in this study was that p21Waf1$^{/Cip1}$ is a potential caspase-3 target during apoptosis (Fig 10.1). This emerged from the finding of a lower molecular weight band (~15KDa) on immunoblots of butyrate-treated LIM1215 cells that

first appeared immediately after onset of increase in DEVD-caspase activity and which then increased thereafter. Its concentration in floating apoptotic cells, as opposed to adherent viable or pre-apoptotic cells, confirms its cleavage during apoptosis, although these studies showed also that cleavage must precede detachment of the cells from the plastic surface of the wells. These findings suggested a search of the primary sequence of p21 for caspase cleavage motifs might be informative. Such a search of the human p21 sequence revealed a likely motif DHVD at a position in the protein that would roughly correspond to the p15 fragment. Following these findings, a number of papers appeared in the literature showing that p21 is cleaved by caspase-3. Interestingly, my search of potential caspase cleavage motifs in other species of p21, revealed that murine and rat p21 contain the same sequence but with the final aspartate replaced by alanine. Such a sequence cannot serve as a site of cleavage for caspases since the final aspartate is critical. It now needs to be confirmed that appearance of p15 does not occur in butyrate-treated rodent CRC.

Two important issues that need addressing are 1) the functional significance of the cleavage of p21 by caspases and 2) the significance of p21 for normal colonocyte homeostasis and colorectal tumorigenesis. Firstly, cleavage of p21 will separate the N-terminal CDK binding site from the C-terminal PCNA-binding site. The former is thought to mediate the inhibitory effects of p21 on apoptosis, while the latter provides a possible nuclear localisation signal. Cleavage might disturb the subcellular distribution of p21/p15 and prevents its apoptosis-inhibitory capacity. The subcellular distribution could be tested by immunocytochemistry for p21/p15 in LIM1215 cells at various intervals after butyrate treatment. Proof that cleavage is important for downstream events of apoptosis could be

tested by examining the effects of butyrate on cells containing mutagenized p21 (where the aspartate is changed for alanine). This would need to be done in cells containing little or no endogenous p21 (such as p21 knock-outs) and where the mutant p21 has been transfected. Studies with rodent cells may be informative since if butyrate-treated rodent CRC undergo apoptosis without cleavage of p21 then the hypothesis that cleavage of p21 is important is unlikely. The transfection experiments in this thesis were designed to test whether over-expression of p21 in LIM1215 or SW480 cells suppresses butyrate-induced apoptosis and whether over-expression of p15 has the opposite effect. The transfections were at least partly successful (since the cells contained the marker EGFP) but no effects on caspase activation or apoptosis were evident. However, this system had its limitations. Overexpression of p21 and p15 appeared to growth arrest the cells, as would be expected, making interpretation of the results difficult. In addition, the transfection efficiencies were rather low and the proportion of tranasfected cells that were apoptotic was too low to make meaningful conclusions about whether p21 and p15 influence butyrate-induced apoptosis. The next experiments should use an inducible system for turning p21/p15 transcription on and off, although even then there are potential problems in the interpretation of the results if the inducing agent (eg tetracycline) directly affects the apoptotic pathway in the cells. The significance of p21 and its cleavage for normal and malignant colonocytes is an area that needs to be explored. An important question is whether p21 is a significant factor in the regulation of apoptosis of these cells. Expression of p21 marks the transition from proliferation to terminal differentiation in intestinal epithelium (Gartel *et al.*, 1996). Cleavage of p21 may, in turn, mark the transition from terminal differentiation to apoptosis at the luminal ends of the crypts. The importance of

caspase-mediated turnover of p21 versus that of transcriptional regulation now needs to be defined. It is possible that cleavage of p21 is a novel regulatory mechanism in apoptotic death of colon cancer cells by butyrate and perhaps other stimuli.

The third major finding of this thesis is that CRC cells could be rendered butyrate-resistant by three cycles of butyrate treatment. There are a number of implications of this. Firstly, it is unlikely that this is simply selecting for those cells which fail to apoptose with butyrate since even 1 mM butyrate (which does not induce apoptosis), was able to induce a partial resistance to 4 mM butyrate. Furthermore, butyrate had to be present continuously for resistance to be maintained. This resistance does not appear to be directly related to the study of Hass (Hass *et al.*, 1997) who found that withdrawal of butyrate in the normal colon results in apoptosis of colonocytes since, in the butyrate-resistant cells, withdrawal of butyrate did not cause apoptosis but rendered the cells sensitive to another application of butyrate. It is more likely that the butyrate is inducing some anti-apoptotic factor. This could be one of the classical survival factors such as Bcl-2 or Bcl-Xl or it could be a factor like p21, Cox or Lox which have their own distinct functions but also exert an anti-apoptotic effect in some systems. Levels of p21 did increase in the butyrate-resistant cells but, curiously, they were also high in the cells that became sensitive to butyrate after withdrawal of this short chain fatty acid for 2 days. This might be explained by some difference in kinetics between p21 induction and reacquisition of butyrate sensitivity. Alternatively, there may be multiple factors involved in the resistance, p21 being just one of them. Unfortunately, the p21 transfection experiments were not conclusive. Further experiments with an inducible transfection

system may enable one to address the question of whether changing the levels of p21 in CRC cells directly influences their sensitivity to butyrate.

Other factors that might be involved in the resistance include the enzymes Cox and Lox. Cox has been shown to suppress apoptosis and promote tumorigenesis in a number of systems. However, an inhibitor of Cox, sulindac sulfide, was unable to induce apoptosis in butyrate-resistant LIM1215 cells, in the presence or absence of butyrate. Similarly, an inhibitor of Lox, NDGA, also did not overcome the resistance. This is despite both of these agents effectively inducing apoptosis by themselves, as well as synergizing with butyrate in inducing apoptosis in sensitive populations of LIM1215 cells.

Understanding the nature of the block in butyrate-resistant cells may provide further clues to the mechanisms of action of butyrate as a pro-apoptotic factor. There are various points in the pathway that might be blocked, leading to resistance. Rapid metabolism of butyrate by the resistant cells cannot be an answer since the resistant cells were also resistant to a structurally-unrelated inhibitor of histone deacetylase TSA, which is not metabolized by these cells. It could be that the histone deacetylase is altered in these cells such that it is no longer affected by butyrate or TSA. However, this would not account for why butyrate needs to be present to maintain the resistance, nor would it explain the cross-resistance with sulindac sulfide, sulindac sulfone and NDGA, which do not act via inhibition of histone deacetylase. Rather, it, suggests that the block is at some later stage. Clearly, this block must be before the stage of DEVD-caspase activation since staurosporine and TPEN were still able to induce DEVD-caspase activation in butyrate-resistant cells. Further experiments should determine which of the known pathways of apoptosis are blocked in butyrate-resistant cells and which are still sensitive.

Finally, the relationship of butyrate resistance in LIM1215 cells to that which might occur in vivo needs investigation. Normal colonocytes may be resistant to butyrate (Hass *et al.*, 1997; Roediger, 1982). There is little known about whether primary CRC tumour cells are naturally resistant. Since both of these types of cell are continuously exposed to mM concentrations of butyrate in vivo, this may be the case. It could be argued that since butyrate is used as a fuel in vivo, the cells need to develop anti-apoptotic mechanisms to block butyrate toxicity. At the same time, the cells should retain sensitivity to other pathways of apoptosis since apoptosis is probably required for the normal turnover of these cells. Understanding how the butyrate pathway is turned off, while other pathways (eg via staurosporine) are maintained may provide clues to mechanisms of apoptosis, in general.

The cross-resistance to sulindac compounds may have therapeutic implications. If CRC tumour cells in vivo are butyrate-resistant, then they may also be resistant to sulindac and some other chemopreventive agents. Good in vitro model systems for studying apoptosis of primary normal and malignant colonic epithelial cells are required. These systems would also enable further testing of the synergistic interactions between dietary and chemopreventive agents in apoptosis of CRC cells. Sulindac is a classical NSAID which were thought to act their antitumour property by inhibition of Cox. Two metabolites of sulindac in vivo are sulindac sulfide, which has an ability to inhibit Cox activity and sulfone, which does not inhibit Cox (Piazza *et al.*, 1997; Thompson *et al.*, 1997). Both sulindac sulfide or sulfone induced apoptosis of colorectal cancer cells in concentration- and time-dependent ways, suggesting sulindac-induced apoptosis is not dependent on inhibition of Cox, although higher concentrations and longer incubation time were

required for sulindac sulfone than for sulfide. There are other pieces of evidence supporting this hypothesis. Firstly, the interaction between sulindac sulfide and AA on activation of caspase-3 was synergistic rather than additive. This was also reported in the study by Chan, et al. (1998). Synergy between these agents would not be expected if sulindac sulfide was simply acting via increase in the intracellular levels of AA. Secondly, SW480 cells lack Cox-2 (Smith *et al.*, 2000), but still undergo apoptosis. Therefore, there are likely to be other mechanisms present. These include effects on PPARδ. It may be that one or more of the genes regulated by PPARδ is a gene whose product suppresses apoptosis in colorectal cancer cells and that by blocking PPARδ, sulindac enhances butyrate-induced apoptosis. The relevance of this pathway to mechanisms of butyrate-induced apoptosis and butyrate resistance now requires further investigation.

The fourth major finding of this thesis is that combination of butyrate and NSAIDs or other agents can result in synergistic or additive effects on apoptosis of CRC cells (Table 10.1). One issue addressed in this thesis concerns potential synergistic interactions between butyrate and Cox-2/Lox inhibitors in apoptosis of colorectal cancer cells in their ability to induce DEVD-caspase activity. This may be important in chemoprevention of colorectal cancers where a combination of sulindac and high dietary fibre may yield better effects than either agent alone (Fig 10.2). The synergy between butyrate and sulindac also suggests that the mechanism of butyrate-induced apoptosis is different from that induced by sulindac sulfide and sulfone.

Another of the synergistic interaction was between butyrate and zinc depletion. One aim of the experiments in this thesis was to investigate the involvement of zinc in the apoptosis of LIM1215 cells. Previous studies have shown that a) intracellular zinc levels

Table 10.1: Synergistic of additive effects between butyrate and other agents on the activation of DEVD-caspase in CRC cells

	Interaction with butyrate	
	Synergistic	Additive
Sulindac sulfide	+	-
Sulindac sulfone	+	-
NDGA	+	-
AA	+	-
TPEN	+	-
Phytic acid	-	+

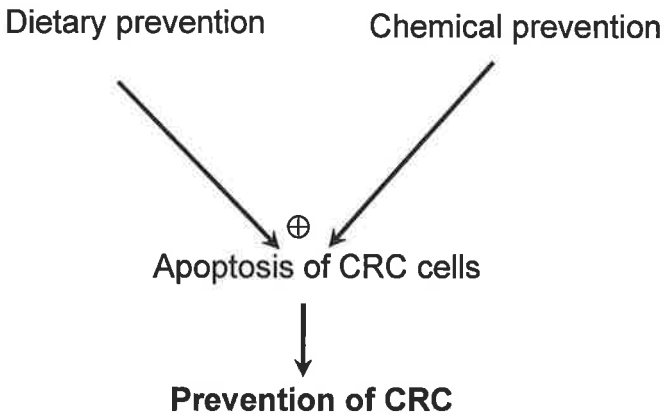


Figure 10.2 Synergy between dietary factors and chemopreventive agents in apoptosis of CRC cells and prevention of CRC.

were higher in colon cancer tissue than the adjacent mucosa (Song *et al.*, 1993), and b) zinc deficiency in vivo is associated with enhanced apoptotic cell death in epithelial tissues and may play an important role in the damage to the gastrointestinal lining and associated diarrhoea. The experiments led to four major conclusions: 1) apoptosis occurs in zinc-depleted cells and is accompanied by activation of caspase-3 and caspase-6; 2) there is only a short lag period (2 h) between depletion of intracellular zinc and onset of caspase-3 activation; 3) zinc depletion enhances butyrate-induced apoptosis while zinc supplementation suppresses it; and, 4) activation of caspase-3 by zinc depletion is rapidly followed by loss of p21, first to a 15KDa fragment which is then further degraded.

The studies with Zinquin confirmed that LIM1215 cells had appreciable levels of labile zinc in their cytoplasm and this zinc was rapidly chelated by TPEN. This pool of zinc is clearly important in the regulation of apoptosis. However, to what extent in vivo zinc deficiency is associated with enhanced rates of caspase activation, p21 degradation and apoptosis in normal and malignant colorectal tissues needs to be determined. This will best be answered in the context of experimental zinc deficiency in animal models of colorectal tumorigenesis.

The findings in TPEN-treated cells of a time- and concentration-dependent increase in DEVD-caspase activity, loss of the 32KDa zymogen form of caspase-3, and partial suppression of DNA fragmentation by caspase peptide inhibitors (including a specific inhibitor of caspase-3, zDEVD-fmk), argue for a major role of caspase-3 in the TPEN-induced apoptosis. This conclusion is strengthened by the recent study of TPEN-induced cell death in human renal cell carcinoma cell lines, where mutant cell lines lacking caspase-3, were resistant to TPEN-induced apoptosis (Kolenko *et al.*, 1999). Since these resistant

cell lines also had variable decreases in caspase-7, caspase-8 and caspase-10, it cannot be ruled out that loss of one of these upstream caspases may also have been important. Caspase-6, which is the caspase responsible for cleavage of nuclear lamins (Takahashi *et al.*, 1996), was also activated in TPEN-treated cells in this study and this caspase may be responsible for the subsequent nuclear fragmentation in zinc-depleted cells. In view of the finding by Song et al (Song *et al.*, 1993) that colon cancers may have increases in the level of zinc, this metal could render them less susceptible to the protective effects of butyrate in vivo. Secondly, physiological factors which act to deplete intracellular zinc (eg phytic acid in high fibre diets), may facilitate the protective effects of butyrate in vivo.

The final interaction study was that between butyrate and another dietary factor, and physiological zinc chelator, phytic acid. Although, I have shown that phytic acid kills colorectal cancer cells in vitro by apoptotic death, in which caspase-3 is involved, apoptosis was not mediated by depletion of intracellular zinc and there is a additive rather than a synergy between butyrate and phytic acid in inducing apoptosis. Like butyrate, the anti-carcinogenic action of phytic acid may be via apoptosis, as well as by inhibition of proliferation and induction of differentiation as previously reported (Yang and Shamsuddin, 1995; Shamsuddin and Yang, 1995; Shamsuddin *et al.*, 1996). The mechanisms by which phytic acid induces apoptosis of CRC need further investigation. Effects on phosphatidylinositol-3' kinase (a known anti-apoptotic factor for primary human epithelial cells (Gire *et al.*, 2000) could be tested, since phytic acid inhibits activation of the Erks family of tyrosine kinases and the activation of transcription factor AP-1 by blocking this enzyme (Huang *et al.*, 1997).

In conclusion, the experiments described in this thesis have provided new data on the role of caspase-3 in butyrate-induced apoptosis of colorectal cancer cells, and the interaction of these factors with a number of cellular factors and pharmacological agents.

BIBLIOGRAPHY

Abarzua, P., LoSardo, J.E., Gubler, M.L. and Neri, A., Microinjection of monoclonal antibody PAb421 into human SW480 colorectal carcinoma cells restores the transcription activation function to mutant p53. *Cancer Res*, **55,** 3490-4 (1995).

Abdel Rahman, W.M., Georgiades, I.B., Curtis, L.J., Arends, M.J. and Wyllie, A.H., Role of BAX mutations in mismatch repair-deficient colorectal carcinogenesis. *Oncogene*, **18,** 2139-42 (1999).

Adams, J.M. and Cory, S., The Bcl-2 protein family: arbiters of cell survival. *Science*, **281,** 1322-6 (1998).

Adolphe, M., Deysson, G. and Lechat, P., Action of some steroid and non-steroid anti-inflammatory agents on the cell cycle: cytophotometric study of the DNA content. *Rev Eur Etud Clin Biol*, **17,** 320-3 (1972).

Agapova, L.S., Ilyinskaya, G.V., Turovets, N.A., Ivanov, A.V., Chumakov, P.M. and Kopnin, B.P., Chromosome changes caused by alterations of p53 expression. *Mutat Res*, **354,** 129-38 (1996).

Ahn, Y.H., Kim, Y.H., Hong, S.H. and Koh, J.Y., Depletion of intracellular zinc induces protein synthesis-dependent neuronal apoptosis in mouse cortical culture. *Exp Neurol*, **154,** 47-56 (1998).

Aiuchi, T., Mihara, S., Nakaya, M., Masuda, Y., Nakajo, S. and Nakaya, K., Zinc ions prevent processing of caspase-3 during apoptosis induced by geranylgeraniol in HL-60 cells. *J Biochem Tokyo*, **124,** 300-3 (1998).

Akao, Y., Otsuki, Y., Kataoka, S., Ito, Y. and Tsujimoto, Y., Multiple subcellular localization of bcl-2: detection in nuclear outer membrane, endoplasmic reticulum membrane, and mitochondrial membranes. *Cancer Res*, **54,** 2468-71 (1994).

Alberts, D.S., Martinez, M.E., Roe, D.J., Guillen Rodriguez, J.M., Marshall, J.R., van Leeuwen, J.B., Reid, M.E., Ritenbaugh, C., Vargas, P.A., Bhattacharyya, A.B., Earnest, D.L. and Sampliner, R.E., Lack of effect of a high-fiber cereal supplement on the recurrence of colorectal adenomas. Phoenix Colon Cancer Prevention Physicians' Network [see comments]. *N Engl J Med*, **342,** 1156-62 (2000).

An, B. and Dou, Q.P., Cleavage of retinoblastoma protein during apoptosis: an interleukin 1 beta-converting enzyme-like protease as candidate. *Cancer Res*, **56,** 438-42 (1996).
Antalis, T.M. and Reeder, J.A., Butyrate regulates gene expression of the plasminogen activating system in colon cancer cells. *Int J Cancer*, **62,** 619-26 (1995).

Archer, S.Y., Meng, S., Shei, A. and Hodin, R.A., p21(WAF1) is required for butyrate-mediated growth inhibition of human colon cancer cells. *Proc Natl Acad Sci U S A*, **95,** 6791-6 (1998).

Arnold, J.T., Wilkinson, B.P., Sharma, S. and Steele, V.E., Evaluation of chemopreventive agents in different mechanistic classes using a rat tracheal epithelial cell culture transformation assay. *Cancer Res*, **55,** 537-43 (1995).

Babich, H., Borenfreund, E. and Stern, A., Comparative cytotoxicities of selected minor dietary non-nutrients with chemopreventive properties. *Cancer Lett*, **73,** 127-33 (1993).
Bai, L. and Merchant, J.L., Transcription factor ZBP-89 cooperates with histone acetyltransferase p300 during butyrate activation of p21waf1 transcription in human cells. *J Biol Chem*, **275,** 30725-33 (2000).

Barbolt, T.A. and Abraham, R., The effect of bran on dimethylhydrazine-induced colon carcinogenesis in the rat. *Proc Soc Exp Biol Med*, **157,** 656-9 (1978).

Barnard, J.A. and Warwick, G., Butyrate rapidly induces growth inhibition and differentiation in HT-29 cells. *Cell Growth Differ*, **4,** 495-501 (1993).

Barnes, C.J. and Lee, M., Chemoprevention of spontaneous intestinal adenomas in the adenomatous polyposis coli Min mouse model with aspirin. *Gastroenterology*, **114,** 873-7 (1998).

Bedi, A., Pasricha, P.J., Akhtar, A.J., Barber, J.P., Bedi, G.C., Giardiello, F.M., Zehnbauer, B.A., Hamilton, S.R. and Jones, R.J., Inhibition of apoptosis during development of colorectal cancer. *Cancer Res*, **55,** 1811-6 (1995).

Bergsma Kadijk, J.A., van 't Veer, P., Kampman, E. and Burema, J., Calcium does not protect against colorectal neoplasia. *Epidemiology*, **7,** 590-7 (1996).

Billon, N., Carlisi, D., Datto, M.B., van Grunsven, L.A., Watt, A., Wang, X.F. and Rudkin, B.B., Cooperation of Sp1 and p300 in the induction of the CDK inhibitor p21WAF1/CIP1 during NGF-mediated neuronal differentiation. *Oncogene*, **18,** 2872-82 (1999).

Bissonnette, N. and Hunting, D.J., p21-induced cycle arrest in G1 protects cells from apoptosis induced by UV-irradiation or RNA polymerase II blockage. *Oncogene*, **16,** 3461-9 (1998).

Blagosklonny, M.V., Wu, G.S., Omura, S. and el Deiry, W.S., Proteasome-dependent regulation of p21WAF1/CIP1 expression. *Biochem Biophys Res Commun*, **227,** 564-9 (1996).

Blanchard, R.K. and Cousins, R.J., Differential display of intestinal mRNAs regulated by dietary zinc. *Proc Natl Acad Sci U S A*, **93,** 6863-8 (1996).

Boffa, L.C., Mariani, M.R. and Parker, M.I., Selective hypermethylation of transcribed nucleosomal DNA by sodium butyrate. *Exp Cell Res*, **211,** 420-3 (1994).

Bogen, K.T., Cancer potencies of heterocyclic amines found in cooked foods. *Food Chem Toxicol*, **32,** 505-15 (1994).

Boland, C.R., Sato, J., Saito, K., Carethers, J.M., Marra, G., Laghi, L. and Chauhan, D.P., Genetic instability and chromosomal aberrations in colorectal cancer: a review of the current models. *Cancer Detect Prev*, **22,** 377-82 (1998).

Boland, C.R., Sinicrope, F.A., Brenner, D.E. and Carethers, J.M., Colorectal cancer prevention and treatment. *Gastroenterology*, **118,** S115-28 (2000).

Boolbol, S.K., Dannenberg, A.J., Chadburn, A., Martucci, C., Guo, X.J., Ramonetti, J.T., Abreu Goris, M., Newmark, H.L., Lipkin, M.L., DeCosse, J.J. and Bertagnolli, M.M., Cyclooxygenase-2 overexpression and tumor formation are blocked by sulindac in a murine model of familial adenomatous polyposis. *Cancer Res*, **56,** 2556-60 (1996).

Borner, C., Martinou, I., Mattmann, C., Irmler, M., Schaerer, E., Martinou, J.C. and Tschopp, J., The protein bcl-2 alpha does not require membrane attachment, but two conserved domains to suppress apoptosis. *J Cell Biol*, **126,** 1059-68 (1994).

Borner, C. and Monney, L., Apoptosis without caspases: an inefficient molecular guillotine? *Cell Death Differ*, **6,** 497-507 (1999).

Bortner, C., Oldenburg, N. and Cidlowski, J., The role of DNA fragmentation in apoptosis. *Trends Cell Bio*, **5,** 21-26 (1995).

Bortuzzo, C., Hanif, R., Kashfi, K., Staiano Coico, L., Shiff, S.J. and Rigas, B., The effect of leukotrienes B and selected HETEs on the proliferation of colon cancer cells. *Biochim Biophys Acta*, **1300,** 240-6 (1996).

Bradbury, E.M., Reversible histone modifications and the chromosome cell cycle. *Bioessays*, **14,** 9-16 (1992).

Bresalier, R.S., Prevention of colorectal cancer: tumor progression, chemoprevention, and COX-2 inhibition. *Gastroenterology*, **119,** 267-8 (2000).

Briggs, D. and Wahlqvist, M., *Food Facts*, 2nd ed., Penguin Books Australia, Ringwood, Victoria (1998).

Buquet Fagot, C., Lallemand, F., Charollais, R.H. and Mester, J., Sodium butyrate inhibits the phosphorylation of the retinoblastoma gene product in mouse fibroblasts by a transcription-dependent mechanism. *J Cell Physiol*, **166,** 631-6 (1996).

Calabresse, C., Venturini, L., Ronco, G., Villa, P., Chomienne, C. and Belpomme, D., Butyric acid and its monosaccharide ester induce apoptosis in the HL-60 cell line. *Biochem Biophys Res Commun*, **195,** 31-8 (1993).

Cassidy, A., Bingham, S.A. and Cummings, J.H., Starch intake and colorectal cancer risk: an international comparison. *Br J Cancer*, **69,** 937-42 (1994).

Cats, A., Kleibeuker, J.H., van der Meer, R., Kuipers, F., Sluiter, W.J., Hardonk, M.J., Oremus, E.T., Mulder, N.H. and de Vries, E.G., Randomized, double-blinded, placebo-controlled intervention study with supplemental calcium in families with hereditary nonpolyposis colorectal cancer. *J Natl Cancer Inst*, **87,** 598-603 (1995).

Cayrol, C., Knibiehler, M. and Ducommun, B., p21 binding to PCNA causes G1 and G2 cell cycle arrest in p53-deficient cells. *Oncogene*, **16,** 311-20 (1998).

Chai, F., Evdokiou, A., Young, G.P. and Zalewski, P.D., Involvement of p21(Waf1/Cip1) and its cleavage by DEVD-caspase during apoptosis of colorectal cancer cells induced by butyrate. *Carcinogenesis*, **21,** 7-14 (2000).

Chai, F., Truong Tran, A.Q., Ho, L.H. and Zalewski, P.D., Regulation of caspase activation and apoptosis by cellular zinc fluxes and zinc deprivation: A review. *Immunol Cell Biol*, **77,** 272-8 (1999).

Chai, F., Truong-Tran, A.Q., Evdokiou, A., Young, G.P. and Zalewski, P.D., Intracellular zinc depletion induces caspase activation and p21Waf1/Cip1 cleavage in human epithelial cell lines. *The Journal of Infectious Diseases*, **182,** S85-92 (2000).

Challa, A., Rao, D.R. and Reddy, B.S., Interactive suppression of aberrant crypt foci induced by azoxymethane in rat colon by phytic acid and green tea. *Carcinogenesis*, **18,** 2023-6 (1997).

Chan, T.A., Morin, P.J., Vogelstein, B. and Kinzler, K.W., Mechanisms underlying nonsteroidal antiinflammatory drug-mediated apoptosis. *Proc Natl Acad Sci U S A*, **95,** 681-6 (1998).

Chen, Y.Q., Duniec, Z.M., Liu, B., Hagmann, W., Gao, X., Shimoji, K., Marnett, L.J., Johnson, C.R. and Honn, K.V., Endogenous 12(S)-HETE production by tumor cells and its role in metastasis. *Cancer Res*, **54,** 1574-9 (1994).

Chi, S., Kitanaka, C., Noguchi, K., Mochizuki, T., Nagashima, Y., Shirouzu, M., Fujita, H., Yoshida, M., Chen, W., Asai, A., Himeno, M., Yokoyama, S. and Kuchino, Y., Oncogenic Ras triggers cell suicide through the activation of a caspase-independent cell death program in human cancer cells. *Oncogene*, **18,** 2281-90 (1999).

Chittenden, T., Flemington, C., Houghton, A.B., Ebb, R.G., Gallo, G.J., Elangovan, B., Chinnadurai, G. and Lutz, R.J., A conserved domain in Bak, distinct from BH1 and BH2, mediates cell death and protein binding functions. *EMBO J*, **14,** 5589-96 (1995).

Cho, K.R. and Vogelstein, B., Suppressor gene alterations in the colorectal adenoma-carcinoma sequence. *J Cell Biochem Suppl*, **16g,** 137-41 (1992).

Chung, D.C. and Rustgi, A.K., DNA mismatch repair and cancer. *Gastroenterology*, **109,** 1685-99 (1995).

Clark, L.C., Combs, G.F., Jr., Turnbull, B.W., Slate, E.H., Chalker, D.K., Chow, J., Davis, L.S., Glover, R.A., Graham, G.F., Gross, E.G., Krongrad, A., Lesher, J.L., Jr., Park, H.K., Sanders, B.B., Jr., Smith, C.L. and Taylor, J.R., Effects of selenium supplementation for cancer prevention in patients with carcinoma of the skin. A randomized controlled trial. Nutritional Prevention of Cancer Study Group [see comments] [published erratum appears in JAMA 1997 May 21;277(19):1520]. *JAMA*, **276,** 1957-63 (1996).

Cohen, J.J. and Duke, R.C., Glucocorticoid activation of a calcium-dependent endonuclease in thymocyte nuclei leads to cell death. *J Immunol*, **132,** 38-42 (1984).

Committee-on-Medical-Aspects-of-Food, Nutritional Aspects of the Development of Cancer, Department of Health Report on Health and Social Subjects, Department of Health, London (1998).

Cortese, J.F., Spannhake, E.W., Eisinger, W., Potter, J.J. and Yang, V.W., The 5-lipoxygenase pathway in cultured human intestinal epithelial cells. *Prostaglandins*, **49,** 155-66 (1995).

Crawford, H.C., Fingleton, B.M., Rudolph Owen, L.A., Goss, K.J., Rubinfeld, B., Polakis, P. and Matrisian, L.M., The metalloproteinase matrilysin is a target of beta-catenin transactivation in intestinal tumors. *Oncogene*, **18,** 2883-91 (1999).

Crew, T.E., Elder, D.J. and Paraskeva, C., A cyclooxygenase-2 (COX-2) selective non-steroidal anti-inflammatory drug enhances the growth inhibitory effect of butyrate in colorectal carcinoma cells expressing COX-2 protein: regulation of COX-2 by butyrate. *Carcinogenesis*, **21,** 69-77 (2000).

Cui, L., Takagi, Y., Wasa, M., Sando, K., Khan, J. and Okada, A., Nitric oxide synthase inhibitor attenuates intestinal damage induced by zinc deficiency in rats. *J Nutr*, **129,** 792-8 (1999).

Da Silva, J.J. and Willianms, R.J., Zinc: Lewis acid catalysis and regulation. *In:* R.J. Williams (ed.), *The Biological Chemistry of the Elements.*, pp. 299-318, Clarendon Press, Oxford (1991).

D'Anna, J.A., Tobey, R.A. and Gurley, L.R., Concentration-dependent effects of sodium butyrate in Chinese hamster cells: cell-cycle progression, inner-histone acetylation, histone H1 dephosphorylation, and induction of an H1-like protein. *Biochemistry*, **19,** 2656-71 (1980).

Darzynkiewicz, Z., Sharpless, T., Staiano Coico, L. and Melamed, M.R., Subcompartments of the G1 phase of cell cycle detected by flow cytometry. *Proc Natl Acad Sci U S A*, **77,** 6696-9 (1980).

Dias, V.C., Wallace, J.L. and Parsons, H.G., Modulation of cellular phospholipid fatty acids and leukotriene B4 synthesis in the human intestinal cell (CaCo-2). *Gut*, **33,** 622-7 (1992).

Dinsdale, D. and Williams, R.B., The enhancement by dietary zinc deficiency of the susceptibility of the rat duodenum to colchicine. *Br J Nutr*, **37,** 135-42 (1977).

Doglioni, C., Pelosio, P., Laurino, L., Macri, E., Meggiolaro, E., Favretti, F. and Barbareschi, M., p21/WAF1/CIP1 expression in normal mucosa and in adenomas and adenocarcinomas of the colon: its relationship with differentiation. *J Pathol*, **179,** 248-53 (1996).

Donato, N.J. and Perez, M., Tumor necrosis factor-induced apoptosis stimulates p53 accumulation and p21WAF1 proteolysis in ME-180 cells. *J Biol Chem*, **273,** 5067-72 (1998).

Donato, N.J., Perez, M., Kang, H., Siddik, Z.H., Ling, Y.H. and Perez Soler, R., EGF receptor and p21WAF1 expression are reciprocally altered as ME-180 cervical carcinoma cells progress from high to low cisplatin sensitivity. *Clin Cancer Res*, **6,** 193-202 (2000).

Dou, Q.P. and An, B., Rb and apoptotic cell death. *Frontiers in Biosciences*, **3,** 419-430 (1998).

Dou, Q.P., An, B. and Will, P.L., Induction of a retinoblastoma phosphatase activity by anticancer drugs accompanies p53-independent G1 arrest and apoptosis. *Proc Natl Acad Sci U S A*, **92,** 9019-23 (1995).

DuBois, R.N., Giardiello, F.M. and Smalley, W.E., Nonsteroidal anti-inflammatory drugs, eicosanoids, and colorectal cancer prevention. *Gastroenterol Clin North Am*, **25,** 773-91 (1996).

Dulic, V., Kaufmann, W.K., Wilson, S.J., Tlsty, T.D., Lees, E., Harper, J.W., Elledge, S.J. and Reed, S.I., p53-dependent inhibition of cyclin-dependent kinase activities in human fibroblasts during radiation-induced G1 arrest. *Cell*, **76,** 1013-23 (1994).

Duval, A., Gayet, J., Zhou, X.P., Iacopetta, B., Thomas, G. and Hamelin, R., Frequent frameshift mutations of the TCF-4 gene in colorectal cancers with microsatellite instability. *Cancer Res*, **59,** 4213-5 (1999).

Earnest, D.L., Holubec, H., Wali, R.K., Jolley, C.S., Bissonette, M., Bhattacharyya, A.K., Roy, H., Khare, S. and Brasitus, T.A., Chemoprevention of azoxymethane-induced colonic carcinogenesis by supplemental dietary ursodeoxycholic acid. *Cancer Res*, **54,** 5071-4 (1994).

Earnshaw, W.C., Martins, L.M. and Kaufmann, S.H., Mammalian caspases: structure, activation, substrates, and functions during apoptosis. *Annu Rev Biochem*, **68,** 383-424 (1999).

Eberhart, C.E., Coffey, R.J., Radhika, A., Giardiello, F.M., Ferrenbach, S. and DuBois, R.N., Up-regulation of cyclooxygenase 2 gene expression in human colorectal adenomas and adenocarcinomas. *Gastroenterology*, **107,** 1183-8 (1994).

el Deiry, W.S., Harper, J.W., O'Connor, P.M., Velculescu, V.E., Canman, C.E., Jackman, J., Pietenpol, J.A., Burrell, M., Hill, D.E., Wang, Y. and et al., WAF1/CIP1 is induced in p53-mediated G1 arrest and apoptosis. *Cancer Res*, **54,** 1169-74 (1994).

Elmes, M.E., Apoptosis in the small intestine of zinc-deficient and fasted rats. *J Pathol*, **123,** 219-23 (1977).

Enari, M., Sakahira, H., Yokoyama, H., Okawa, K., Iwamatsu, A. and Nagata, S., A caspase-activated DNase that degrades DNA during apoptosis, and its inhibitor ICAD [see comments] [published erratum appears in Nature 1998 May 28;393(6683):396]. *Nature*, **391,** 43-50 (1998).

Estensen, R.D. and Wattenberg, L.W., Studies of chemopreventive effects of myo-inositol on benzo[a]pyrene-induced neoplasia of the lung and forestomach of female A/J mice. *Carcinogenesis*, **14,** 1975-7 (1993).

Fadok, V.A., Voelker, D.R., Campbell, P.A., Cohen, J.J., Bratton, D.L. and Henson, P.M., Exposure of phosphatidylserine on the surface of apoptotic lymphocytes triggers specific recognition and removal by macrophages. *J Immunol*, 148, 2207-16 (1992).

Fearon, E.R. and Vogelstein, B., A genetic model for colorectal tumorigenesis. *Cell*, **61,** 759-67 (1990).

Fesus, L., Madi, A., Balajthy, Z., Nemes, Z. and Szondy, Z., Transglutaminase induction by various cell death and apoptosis pathways. *Experientia*, 52, 942-9 (1996).

Fesus, L., Tarcsa, E., Kedei, N., Autuori, F. and Piacentini, M., Degradation of cells dying by apoptosis leads to accumulation of epsilon(gamma-glutamyl)lysine isodipeptide in culture fluid and blood. *FEBS Lett*, 284, 109-12 (1991).

Filippovich, I., Sorokina, N., Khanna, K.K. and Lavin, M.F., Butyrate induced apoptosis in lymphoid cells preceded by transient over-expression of HSP70 mRNA. *Biochem Biophys Res Commun*, **198,** 257-65 (1994).

Flores Rozas, H., Kelman, Z., Dean, F.B., Pan, Z.Q., Harper, J.W., Elledge, S.J., O'Donnell, M. and Hurwitz, J., Cdk-interacting protein 1 directly binds with proliferating cell nuclear

antigen and inhibits DNA replication catalyzed by the DNA polymerase delta holoenzyme. *Proc Natl Acad Sci U S A*, **91,** 8655-9 (1994).

Fraker, P.J. and Telford, W.G., A reappraisal of the role of zinc in life and death decisions of cells. *Proc Soc Exp Biol Med*, **215,** 229-36 (1997).

Frankfurt, O.S., Robb, J.A., Sugarbaker, E.V. and Villa, L., Monoclonal antibody to single-stranded DNA is a specific and sensitive cellular marker of apoptosis. *Exp Cell Res*, 226, 387-97 (1996).

Freeman, H.J., Spiller, G.A. and Kim, Y.S., A double-blind study on the effects of differing purified cellulose and pectin fiber diets on 1,2-dimethylhydrazine-induced rat colonic neoplasia. *Cancer Res*, **40,** 2661-5 (1980).

Fukamachi, Y., Karasaki, Y., Sugiura, T., Itoh, H., Abe, T., Yamamura, K. and Higashi, K., Zinc suppresses apoptosis of U937 cells induced by hydrogen peroxide through an increase of the Bcl-2/Bax ratio. *Biochem Biophys Res Commun*, **246,** 364-9 (1998).

Gann, P.H., Manson, J.E., Glynn, R.J., Buring, J.E. and Hennekens, C.H., Low-dose aspirin and incidence of colorectal tumors in a randomized trial [see comments]. *J Natl Cancer Inst*, **85,** 1220-4 (1993).

Gao, X., Grignon, D.J., Chbihi, T., Zacharek, A., Chen, Y.Q., Sakr, W., Porter, A.T., Crissman, J.D., Pontes, J.E., Powell, I.J. and et al., Elevated 12-lipoxygenase mRNA expression correlates with advanced stage and poor differentiation of human prostate cancer. *Urology*, **46,** 227-37 (1995).

Garrido, R., Malecki, A., Hennig, B. and Toborek, M., Nicotine attenuates arachidonic acid-induced neurotoxicity in cultured spinal cord neurons. *Brain Res*, **861,** 59-68 (2000).

Gartel, A.L., Serfas, M.S., Gartel, M., Goufman, E., Wu, G.S., el Deiry, W.S. and Tyner, A.L., p21 (WAF1/CIP1) expression is induced in newly nondividing cells in diverse epithelia and during differentiation of the Caco-2 intestinal cell line. *Exp Cell Res*, **227,** 171-81 (1996).

Gartel, A.L. and Tyner, A.L., Transcriptional regulation of the p21WAF1/CIP1 gene. *Exp Cell Res*, **246,** 280-9 (1999).

Gavrieli, Y., Sherman, Y. and Ben Sasson, S.A., Identification of programmed cell death in situ via specific labeling of nuclear DNA fragmentation. *J Cell Biol*, **119,** 493-501 (1992).

Gerhardsson de Verdier, M., Hagman, U., Peters, R.K., Steineck, G. and Overvik, E., Meat, cooking methods and colorectal cancer: a case-referent study in Stockholm. *Int J Cancer*, **49,** 520-5 (1991).

Gervais, J.L., Seth, P. and Zhang, H., Cleavage of CDK inhibitor p21(Cip1/Waf1) by caspases is an early event during DNA damage-induced apoptosis. *J Biol Chem*, **273,** 19207-12 (1998).

Ghosh, J. and Myers, C.E., Arachidonic acid stimulates prostate cancer cell growth: critical role of 5-lipoxygenase. *Biochem Biophys Res Commun*, **235,** 418-23 (1997).

Giardiello, F.M., Hamilton, S.R., Krush, A.J., Piantadosi, S., Hylind, L.M., Celano, P., Booker, S.V., Robinson, C.R. and Offerhaus, G.J., Treatment of colonic and rectal adenomas with sulindac in familial adenomatous polyposis. *N Engl J Med*, **328,** 1313-6 (1993).

Gibson, P.R., Rosella, O., Rosella, G. and Young, G.P., Butyrate is a potent inhibitor of urokinase secretion by normal colonic epithelium in vitro. *Gastroenterology*, **107,** 410-9 (1994).

Gibson, P.R., Rosella, O., Wilson, A.J., Mariadason, J.M., Rickard, K., Byron, K. and Barkla, D.H., Colonic epithelial cell activation and the paradoxical effects of butyrate. *Carcinogenesis*, **20,** 539-44 (1999).

Giovannucci, E., Egan, K.M., Hunter, D.J., Stampfer, M.J., Colditz, G.A., Willett, W.C. and Speizer, F.E., Aspirin and the risk of colorectal cancer in women [see comments]. *N Engl J Med*, **333,** 609-14 (1995).

Giovannucci, E., Rimm, E.B., Stampfer, M.J., Colditz, G.A., Ascherio, A. and Willett, W.C., Aspirin use and the risk for colorectal cancer and adenoma in male health professionals [see comments]. *Ann Intern Med*, **121,** 241-6 (1994).

Giovannucci, E., Rimm, E.B., Stampfer, M.J., Colditz, G.A., Ascherio, A. and Willett, W.C., Intake of fat, meat, and fiber in relation to risk of colon cancer in men. *Cancer Res*, **54,** 2390-7 (1994).

Giovannucci, E., Stampfer, M.J., Colditz, G.A., Hunter, D.J., Fuchs, C., Rosner, B.A., Speizer, F.E. and Willett, W.C., Multivitamin use, folate, and colon cancer in women in the Nurses' Health Study [see comments]. *Ann Intern Med*, **129,** 517-24 (1998).

Gire, V., Marshall, C. and Wynford Thomas, D., PI-3-kinase is an essential anti-apoptotic effector in the proliferative response of primary human epithelial cells to mutant RAS. *Oncogene*, **19,** 2269-76 (2000).

Gope, R. and Gope, M.L., Effect of sodium butyrate on the expression of retinoblastoma (RB1) and P53 gene and phosphorylation of retinoblastoma protein in human colon tumor cell line HT29. *Cell Mol Biol Noisy le grand*, **39,** 589-97 (1993).

Gorospe, M., Cirielli, C., Wang, X., Seth, P., Capogrossi, M.C. and Holbrook, N.J., p21(Waf1/Cip1) protects against p53-mediated apoptosis of human melanoma cells. *Oncogene*, **14,** 929-35 (1997).

Gorospe, M., Wang, X., Guyton, K.Z. and Holbrook, N.J., Protective role of p21(Waf1/Cip1) against prostaglandin A2-mediated apoptosis of human colorectal carcinoma cells. *Mol Cell Biol*, **16,** 6654-60 (1996).

Greenberg, E.R., Baron, J.A., Tosteson, T.D., Freeman, D.H., Jr., Beck, G.J., Bond, J.H., Colacchio, T.A., Coller, J.A., Frankl, H.D., Haile, R.W. and et al., A clinical trial of antioxidant vitamins to prevent colorectal adenoma. Polyp Prevention Study Group [see comments]. *N Engl J Med*, **331,** 141-7 (1994).

Gu, Y., Sarnecki, C., Aldape, R.A., Livingston, D.J. and Su, M.S., Cleavage of poly(ADP-ribose) polymerase by interleukin-1 beta converting enzyme and its homologs TX and Nedd-2. *J Biol Chem*, 270, 18715-8 (1995).

Gupta, S., Alpini, G., Vemuru, R.P., Hurston, E. and Shafritz, D.A., Butyrate synchronization of hepatocytes: modulation of cycling and cell cycle regulated gene expression. *Growth Factors*, **10,** 171-80 (1994).

Gupta, S.K., Shukla, V.K., Vaidya, M.P., Roy, S.K. and Gupta, S., Serum and tissue trace elements in colorectal cancer. *J Surg Oncol*, **52,** 172-5 (1993).

Ha, H.C., Woster, P.M. and Casero, R.A., Jr., Unsymmetrically substituted polyamine analogue induces caspase-independent programmed cell death in Bcl-2-overexpressing cells. *Cancer Res*, **58,** 2711-4 (1998).

Haenszel, W., Berg, J.W., Segi, M., Kurihara, M. and Locke, F.B., Large-bowel cancer in Hawaiian Japanese. *J Natl Cancer Inst*, **51,** 1765-79 (1973).

Hague, A., Diaz, G.D., Hicks, D.J., Krajewski, S., Reed, J.C. and Paraskeva, C., bcl-2 and bak may play a pivotal role in sodium butyrate-induced apoptosis in colonic epithelial cells; however overexpression of bcl-2 does not protect against bak-mediated apoptosis. *Int J Cancer*, **72,** 898-905 (1997).

Hague, A., Elder, D.J., Hicks, D.J. and Paraskeva, C., Apoptosis in colorectal tumour cells: induction by the short chain fatty acids butyrate, propionate and acetate and by the bile salt deoxycholate. *Int J Cancer*, **60,** 400-6 (1995).

Hague, A., Manning, A.M., Hanlon, K.A., Huschtscha, L.I., Hart, D. and Paraskeva, C., Sodium butyrate induces apoptosis in human colonic tumour cell lines in a p53-independent pathway: implications for the possible role of dietary fibre in the prevention of large-bowel cancer. *Int J Cancer*, **55,** 498-505 (1993).

Hague, A., Moorghen, M., Hicks, D., Chapman, M. and Paraskeva, C., BCL-2 expression in human colorectal adenomas and carcinomas. *Oncogene*, **9,** 3367-70 (1994).

Hanif, R., Pittas, A., Feng, Y., Koutsos, M.I., Qiao, L., Staiano Coico, L., Shiff, S.I. and Rigas, B., Effects of nonsteroidal anti-inflammatory drugs on proliferation and on induction of apoptosis in colon cancer cells by a prostaglandin-independent pathway. *Biochem Pharmacol*, **52,** 237-45 (1996).

Harper, J.W., Adami, G.R., Wei, N., Keyomarsi, K. and Elledge, S.J., The p21 Cdk-interacting protein Cip1 is a potent inhibitor of G1 cyclin-dependent kinases. *Cell*, **75,** 805-16 (1993).

Harper, J.W., Elledge, S.J., Keyomarsi, K., Dynlacht, B., Tsai, L.H., Zhang, P., Dobrowolski, S., Bai, C., Connell Crowley, L., Swindell, E. and et al., Inhibition of cyclin-dependent kinases by p21. *Mol Biol Cell*, **6,** 387-400 (1995).

Hass, R., Busche, R., Luciano, L., Reale, E. and Engelhardt, W.V., Lack of butyrate is associated with induction of Bax and subsequent apoptosis in the proximal colon of guinea pig [comment]. *Gastroenterology*, **112,** 875-81 (1997).

He, T.C., Chan, T.A., Vogelstein, B. and Kinzler, K.W., PPARdelta is an APC-regulated target of nonsteroidal anti-inflammatory drugs. *Cell*, **99,** 335-45 (1999).

Heerdt, B.G., Houston, M.A., Anthony, G.M. and Augenlicht, L.H., Mitochondrial membrane potential (delta psi(mt)) in the coordination of p53-independent proliferation and apoptosis pathways in human colonic carcinoma cells. *Cancer Res*, **58,** 2869-75 (1998).

Heerdt, B.G., Houston, M.A. and Augenlicht, L.H., Short-chain fatty acid-initiated cell cycle arrest and apoptosis of colonic epithelial cells is linked to mitochondrial function. *Cell Growth Differ*, **8,** 523-32 (1997).

Hial, V., Horakova, Z., Shaff, F.E. and Beaven, M.A., Alteration of tumor growth by aspirin and indomethacin: studies with two transplantable tumors in mouse. *Eur J Pharmacol*, **37,** 367-76 (1976).

Hirata, M. and Murad, F., Interrelationships of cyclic GMP, inositol phosphates, and calcium. *Adv Pharmacol*, **26,** 195-216 (1994).

Hirose, M., Hoshiya, T., Akagi, K., Futakuchi, M. and Ito, N., Inhibition of mammary gland carcinogenesis by green tea catechins and other naturally occurring antioxidants in female Sprague-Dawley rats pretreated with 7,12-dimethylbenz[alpha]anthracene. *Cancer Lett*, **83,** 149-56 (1994).

Hirose, M., Ozaki, K., Takaba, K., Fukushima, S., Shirai, T. and Ito, N., Modifying effects of the naturally occurring antioxidants gamma-oryzanol, phytic acid, tannic acid and n-

tritriacontane-16, 18-dione in a rat wide-spectrum organ carcinogenesis model. *Carcinogenesis*, **12,** 1917-21 (1991).

Hirose, Y., Yoshimi, N., Suzui, M., Kawabata, K., Tanaka, T. and Mori, H., Expression of bcl-2, bax, and bcl-XL proteins in azoxymethane-induced rat colonic adenocarcinomas. *Mol Carcinog*, **19,** 25-30 (1997).

Hockenbery, D., Nunez, G., Milliman, C., Schreiber, R.D. and Korsmeyer, S.J., Bcl-2 is an inner mitochondrial membrane protein that blocks programmed cell death. *Nature*, **348,** 334-6 (1990).

Hofmann, K., Bucher, P. and Tschopp, J., The CARD domain: a new apoptotic signalling motif. *Trends Biochem Sci*, **22,** 155-6 (1997).

Honn, K.V., Tang, D.G., Gao, X., Butovich, I.A., Liu, B., Timar, J. and Hagmann, W., 12-lipoxygenases and 12(S)-HETE: role in cancer metastasis. *Cancer Metastasis Rev*, **13,** 365-96 (1994).

Honn, K.V., Timar, J., Rozhin, J., Bazaz, R., Sameni, M., Ziegler, G. and Sloane, B.F., A lipoxygenase metabolite, 12-(S)-HETE, stimulates protein kinase C-mediated release of cathepsin B from malignant cells. *Exp Cell Res*, **214,** 120-30 (1994).

Hotz, M.A., Traganos, F. and Darzynkiewicz, Z., Changes in nuclear chromatin related to apoptosis or necrosis induced by the DNA topoisomerase II inhibitor fostriecin in MOLT-4 and HL-60 cells are revealed by altered DNA sensitivity to denaturation. *Exp Cell Res*, 201, 184-91 (1992).

Howe, G.R., Benito, E., Castelleto, R., Cornee, J., Esteve, J., Gallagher, R.P., Iscovich, J.M., Deng ao, J., Kaaks, R., Kune, G.A. and et al., Dietary intake of fiber and decreased risk of cancers of the colon and rectum: evidence from the combined analysis of 13 case-control studies [see comments]. *J Natl Cancer Inst*, **84,** 1887-96 (1992).

Hronek, M., Zadak, Z., Solichova, D., Jandik, P. and Melichar, B., The association between specific nutritional antioxidants and manifestation of colorectal cancer. *Nutrition*, **16,** 189-91 (2000).

Huang, C., Ma, W.Y., Hecht, S.S. and Dong, Z., Inositol hexaphosphate inhibits cell transformation and activator protein 1 activation by targeting phosphatidylinositol-3' kinase [published erratum appears in Cancer Res 1997 Nov 15;57(22):5198]. *Cancer Res*, **57,** 2873-8 (1997).

Huang, D.C., Adams, J.M. and Cory, S., The conserved N-terminal BH4 domain of Bcl-2 homologues is essential for inhibition of apoptosis and interaction with CED-4. *EMBO J*, **17,** 1029-39 (1998).

Huang, H., Reed, C.P., Zhang, J.S., Shridhar, V., Wang, L. and Smith, D.I., Carboxypeptidase A3 (CPA3): a novel gene highly induced by histone deacetylase inhibitors during differentiation of prostate epithelial cancer cells. *Cancer Res*, **59,** 2981-8 (1999).

Hussey, H.J. and Tisdale, M.J., Inhibition of tumour growth by lipoxygenase inhibitors. *Br J Cancer*, **74,** 683-7 (1996).

Hyman, J., Baron, J.A., Dain, B.J., Sandler, R.S., Haile, R.W., Mandel, J.S., Mott, L.A. and Greenberg, E.R., Dietary and supplemental calcium and the recurrence of colorectal adenomas. *Cancer Epidemiol Biomarkers Prev*, **7,** 291-5 (1998).

Iqbal, T.H., Lewis, K.O. and Cooper, B.T., Phytase activity in the human and rat small intestine. *Gut*, **35,** 1233-6 (1994).

Iseki, K., Tatsuta, M., Uehara, H., Iishi, H., Yano, H., Sakai, N. and Ishiguro, S., Inhibition of angiogenesis as a mechanism for inhibition by 1alpha-hydroxyvitamin D3 and 1,25-dihydroxyvitamin D3 of colon carcinogenesis induced by azoxymethane in Wistar rats. *Int J Cancer*, **81,** 730-3 (1999).

Ishijima, N., Miki, C., Ishida, T., Kinoshita, T. and Suzuki, H., The immunohistochemical expression of BCL-2 oncoprotein in colorectal adenocarcinoma. *Surg Today*, **29,** 682-4 (1999).

Ito, N., Hasegawa, R., Sano, M., Tamano, S., Esumi, H., Takayama, S. and Sugimura, T., A new colon and mammary carcinogen in cooked food, 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP). *Carcinogenesis*, **12,** 1503-6 (1991).

Jacoby, R.F., Marshall, D.J., Newton, M.A., Novakovic, K., Tutsch, K., Cole, C.E., Lubet, R.A., Kelloff, G.J., Verma, A., Moser, A.R. and Dove, W.F., Chemoprevention of spontaneous intestinal adenomas in the Apc Min mouse model by the nonsteroidal anti-inflammatory drug piroxicam. *Cancer Res*, **56,** 710-4 (1996).

Janson, W., Brandner, G. and Siegel, J., Butyrate modulates DNA-damage-induced p53 response by induction of p53-independent differentiation and apoptosis. *Oncogene*, **15,** 1395-406 (1997).

Janssen, A.M., Bosman, C.B., Kruidenier, L., Griffioen, G., Lamers, C.B., van Krieken, J.H., van de Velde, C.J. and Verspaget, H.W., Superoxide dismutases in the human colorectal cancer sequence. *J Cancer Res Clin Oncol*, **125,** 327-35 (1999).

Janssen, A.M., Bosman, C.B., Sier, C.F., Griffioen, G., Kubben, F.J., Lamers, C.B., van Krieken, J.H., van de Velde, C.J. and Verspaget, H.W., Superoxide dismutases in relation to the overall survival of colorectal cancer patients. *Br J Cancer*, **78,** 1051-7 (1998).

Jenab, M. and Thompson, L.U., The influence of phytic acid in wheat bran on early biomarkers of colon carcinogenesis. *Carcinogenesis*, **19,** 1087-92 (1998).

Jenab, M. and Thompson, L.U., Phytic acid in wheat bran affects colon morphology, cell differentiation and apoptosis. *Carcinogenesis*, **21,** 1547-52 (2000).

Jones, B.E., Lo, C.R., Srinivasan, A., Valentino, K.L. and Czaja, M.J., Ceramide induces caspase-independent apoptosis in rat hepatocytes sensitized by inhibition of RNA synthesis. *Hepatology*, **30,** 215-22 (1999).

Kamano, T., Mikami, Y., Kurasawa, T., Tsurumaru, M., Matsumoto, M., Kano, M. and Motegi, K., Ratio of primary and secondary bile acids in feces: possible marker for colorectal cancer? *Dis Colon Rectum*, **42,** 668-72 (1999).

Kamitani, H., Geller, M. and Eling, T., Expression of 15-lipoxygenase by human colorectal carcinoma Caco-2 cells during apoptosis and cell differentiation. *J Biol Chem*, **273,** 21569-77 (1998).

Kanavaros, P., Stefanaki, K., Valassiadou, K., Vlachonikolis, J., Mavromanolakis, M., Vlychou, M., Kakolyris, S., Gorgoulis, V., Tzardi, M. and Georgoulias, V., Expression of p53, p21/waf, bcl-2, bax, Rb and Ki67 proteins in colorectal adenocarcinomas. *Med Oncol*, **16,** 23-30 (1999).

Kar, S., Quirion, R. and Parent, A., An interaction between inositol hexakisphosphate (IP6) and insulin-like growth factor II receptor binding sites in the rat brain. *Neuroreport*, **5,** 625-8 (1994).

Kastner, P., Mark, M. and Chambon, P., Nonsteroid nuclear receptors: what are genetic studies telling us about their role in real life? *Cell*, **83,** 859-69 (1995).

Kaufman, H.W. and Kleinberg, I., Effect of pH on calcium binding by phytic acid and its inositol phosphoric acid derivatives and on the solubility of their calcium salts. *Arch Oral Biol*, **16,** 445-60 (1971).

Kawamori, T., Lubet, R., Steele, V.E., Kelloff, G.J., Kaskey, R.B., Rao, C.V. and Reddy, B.S., Chemopreventive effect of curcumin, a naturally occurring anti-inflammatory agent, during the promotion/progression stages of colon cancer. *Cancer Res*, **59,** 597-601 (1999).

Keller, J.J., Offerhaus, G.J., Polak, M., Goodman, S.N., Zahurak, M.L., Hylind, L.M., Hamilton, S.R. and Giardiello, F.M., Rectal epithelial apoptosis in familial adenomatous polyposis patients treated with sulindac [see comments]. *Gut*, **45,** 822-8 (1999).

Key, T.J., Fraser, G.E., Thorogood, M., Appleby, P.N., Beral, V., Reeves, G., Burr, M.L., Chang Claude, J., Frentzel Beyme, R., Kuzma, J.W., Mann, J. and McPherson, K., Mortality in vegetarians and non-vegetarians: a collaborative analysis of 8300 deaths among 76,000 men and women in five prospective studies. *Public Health Nutr*, **1,** 33-41 (1998).

Kim, Y.I., Salomon, R.N., Graeme Cook, F., Choi, S.W., Smith, D.E., Dallal, G.E. and Mason, J.B., Dietary folate protects against the development of macroscopic colonic neoplasia in a dose responsive manner in rats. *Gut*, **39,** 732-40 (1996).

King, K.L. and Cidlowski, J.A., Cell cycle regulation and apoptosis. *Annu Rev Physiol*, **60,** 601-17 (1998).

Kinzler, K.W. and Vogelstein, B., Lessons from hereditary colorectal cancer. *Cell*, **87,** 159-70 (1996).

Kitanaka, C. and Kuchino, Y., Caspase-independent programmed cell death with necrotic morphology. *Cell Death Differ*, **6,** 508-15 (1999).

Kivinen, L., Tsubari, M., Haapajarvi, T., Datto, M.B., Wang, X.F. and Laiho, M., Ras induces p21Cip1/Waf1 cyclin kinase inhibitor transcriptionally through Sp1-binding sites. *Oncogene*, **18,** 6252-61 (1999).

Kluck, R.M., Bossy Wetzel, E., Green, D.R. and Newmeyer, D.D., The release of cytochrome c from mitochondria: a primary site for Bcl-2 regulation of apoptosis [see comments]. *Science*, **275,** 1132-6 (1997).

Kolenko, V., Uzzo, R.G., Bukowski, R., Bander, N.H., Novick, A.C., Hsi, E.D. and Finke, J.H., Dead or dying: necrosis versus apoptosis in caspase-deficient human renal cell carcinoma. *Cancer Res*, **59,** 2838-42 (1999).

Kook, S., Shim, S.R., Choi, S.J., Ahnn, J., Kim, J.I., Eom, S.H., Jung, Y.K., Paik, S.G. and Song, W.K., Caspase-mediated cleavage of p130cas in etoposide-induced apoptotic Rat-1 cells. *Mol Biol Cell*, **11,** 929-39 (2000).

Korsmeyer, S.J., Shutter, J.R., Veis, D.J., Merry, D.E. and Oltvai, Z.N., Bcl-2/Bax: a rheostat that regulates an anti-oxidant pathway and cell death. *Semin Cancer Biol*, **4,** 327-32 (1993).

Krajewska, M., Moss, S.F., Krajewski, S., Song, K., Holt, P.R. and Reed, J.C., Elevated expression of Bcl-X and reduced Bak in primary colorectal adenocarcinomas. *Cancer Res*, **56,** 2422-7 (1996).

Krajewski, S., Krajewska, M. and Reed, J.C., Immunohistochemical analysis of in vivo patterns of Bak expression, a proapoptotic member of the Bcl-2 protein family. *Cancer Res*, **56,** 2849-55 (1996).

Krajewski, S., Tanaka, S., Takayama, S., Schibler, M.J., Fenton, W. and Reed, J.C., Investigation of the subcellular distribution of the bcl-2 oncoprotein: residence in the nuclear envelope, endoplasmic reticulum, and outer mitochondrial membranes. *Cancer Res*, **53,** 4701-14 (1993).

Kruh, J., Effects of sodium butyrate, a new pharmacological agent, on cells in culture. *Mol Cell Biochem*, **42,** 65-82 (1982).

Kune, G.A., *Causes and control of colorectal cancer: a model for cancer prevention*, KLuwer Academic Publishers, Boston, Dordrecht, London (1996).

Kune, G.A., Kune, S. and Watson, L.F., Colorectal cancer risk, chronic illnesses, operations, and medications: case control results from the Melbourne Colorectal Cancer Study. *Cancer Res*, **48,** 4399-404 (1988).

La Vecchia, C., Negri, E., Decarli, A., D'Avanzo, B., Gallotti, L., Gentile, A. and Franceschi, S., A case-control study of diet and colo-rectal cancer in northern Italy. *Int J Cancer*, **41,** 492-8 (1988).

Lazebnik, Y.A., Kaufmann, S.H., Desnoyers, S., Poirier, G.G. and Earnshaw, W.C., Cleavage of poly(ADP-ribose) polymerase by a proteinase with properties like ICE. *Nature*, 371, 346-7 (1994).

Lee, D.Y., Hayes, J.J., Pruss, D. and Wolffe, A.P., A positive role for histone acetylation in transcription factor access to nucleosomal DNA. *Cell*, **72,** 73-84 (1993).

Lee, H.P., Gourley, L., Duffy, S.W., Esteve, J., Lee, J. and Day, N.E., Colorectal cancer and diet in an Asian population--a case-control study among Singapore Chinese. *Int J Cancer*, **43,** 1007-16 (1989).

Leonardos, L., Butler, L.M., Hewett, P.J., Zalewski, P.D. and Cowled, P.A., The activity of caspase-3-like proteases is elevated during the development of colorectal carcinoma. *Cancer Lett*, **143,** 29-35 (1999).

Levkau, B., Koyama, H., Raines, E.W., Clurman, B.E., Herren, B., Orth, K., Roberts, J.M. and Ross, R., Cleavage of p21Cip1/Waf1 and p27Kip1 mediates apoptosis in endothelial cells through activation of Cdk2: role of a caspase cascade. *Mol Cell*, **1,** 553-63 (1998).

Li, J.M., Datto, M.B., Shen, X., Hu, P.P., Yu, Y. and Wang, X.F., Sp1, but not Sp3, functions to mediate promoter activation by TGF-beta through canonical Sp1 binding sites. *Nucleic Acids Res*, **26,** 2449-56 (1998).

Li, R., Waga, S., Hannon, G.J., Beach, D. and Stillman, B., Differential effects by the p21 CDK inhibitor on PCNA-dependent DNA replication and repair. *Nature*, **371,** 534-7 (1994).

Litvak, D.A., Evers, B.M., Hwang, K.O., Hellmich, M.R., Ko, T.C. and Townsend, C.M., Jr., Butyrate-induced differentiation of Caco-2 cells is associated with apoptosis and early induction of p21Waf1/Cip1 and p27Kip1. *Surgery*, **124,** 161-9 (1998).

Liu, B., Maher, R.J., Hannun, Y.A., Porter, A.T. and Honn, K.V., 12(S)-HETE enhancement of prostate tumor cell invasion: selective role of PKC alpha. *J Natl Cancer Inst*, **86,** 1145-51 (1994).

Liu, B., Parsons, R., Papadopoulos, N., Nicolaides, N.C., Lynch, H.T., Watson, P., Jass, J.R., Dunlop, M., Wyllie, A., Peltomaki, P., de la Chapelle, A., Hamilton, S.R., Vogelstein, B. and Kinzler, K.W., Analysis of mismatch repair genes in hereditary non-polyposis colorectal cancer patients [see comments]. *Nat Med*, **2,** 169-74 (1996).

Liu, B., Timar, J., Howlett, J., Diglio, C.A. and Honn, K.V., Lipoxygenase metabolites of arachidonic and linoleic acids modulate the adhesion of tumor cells to endothelium via regulation of protein kinase C. *Cell Regul*, **2,** 1045-55 (1991).

Liu, X., Kim, C.N., Pohl, J. and Wang, X., Purification and characterization of an interleukin-1beta-converting enzyme family protease that activates cysteine protease P32 (CPP32). *J Biol Chem*, **271,** 13371-6 (1996).

Liu, X., Kim, C.N., Yang, J., Jemmerson, R. and Wang, X., Induction of apoptotic program in cell-free extracts: requirement for dATP and cytochrome c. *Cell*, **86,** 147-57 (1996).

Liu, X., Li, P., Widlak, P., Zou, H., Luo, X., Garrard, W.T. and Wang, X., The 40-kDa subunit of DNA fragmentation factor induces DNA fragmentation and chromatin condensation during apoptosis. *Proc Natl Acad Sci U S A*, **95,** 8461-6 (1998).

Liu, X., Zou, H., Slaughter, C. and Wang, X., DFF, a heterodimeric protein that functions downstream of caspase-3 to trigger DNA fragmentation during apoptosis. *Cell*, **89,** 175-84 (1997).

Lonnerdal, B., Bell, J.G., Hendrickx, A.G., Burns, R.A. and Keen, C.L., Effect of phytate removal on zinc absorption from soy formula. *Am J Clin Nutr*, **48,** 1301-6 (1988).

Lu, X., Xie, W., Reed, D., Bradshaw, W.S. and Simmons, D.L., Nonsteroidal antiinflammatory drugs cause apoptosis and induce cyclooxygenases in chicken embryo fibroblasts. *Proc Natl Acad Sci U S A*, **92,** 7961-5 (1995).

Lu, Y., Yamagishi, N., Yagi, T. and Takebe, H., Mutated p21(WAF1/CIP1/SDI1) lacking CDK-inhibitory activity fails to prevent apoptosis in human colorectal carcinoma cells. *Oncogene*, **16,** 705-12 (1998).

MacDonald, R.S., Park, J.H. and Thornton, W.H., Jr., Insulin, IGF-1, and IGF-2 receptors in rat small intestine following massive small bowel resection. Analysis by binding, flow cytometry, and immunohistochemistry. *Dig Dis Sci*, **38,** 1658-69 (1993).

Magalova, T., Bella, V., Brtkova, A., Beno, I., Kudlackova, M. and Volkovova, K., Copper, zinc and superoxide dismutase in precancerous, benign diseases and gastric, colorectal and breast cancer. *Neoplasma*, **46,** 100-4 (1999).

Mahmoud, N.N., Boolbol, S.K., Dannenberg, A.J., Mestre, J.R., Bilinski, R.T., Martucci, C., Newmark, H.L., Chadburn, A. and Bertagnolli, M.M., The sulfide metabolite of sulindac prevents tumors and restores enterocyte apoptosis in a murine model of familial adenomatous polyposis. *Carcinogenesis*, **19,** 87-91 (1998).

Malkhosyan, S., Rampino, N., Yamamoto, H. and Perucho, M., Frameshift mutator mutations [letter]. *Nature*, **382,** 499-500 (1996).

Mangelsdorf, D.J., Thummel, C., Beato, M., Herrlich, P., Schutz, G., Umesono, K., Blumberg, B., Kastner, P., Mark, M., Chambon, P. and et al., The nuclear receptor superfamily: the second decade. *Cell*, **83,** 835-9 (1995).

Mann, B., Gelos, M., Siedow, A., Hanski, M.L., Gratchev, A., Ilyas, M., Bodmer, W.F., Moyer, M.P., Riecken, E.O., Buhr, H.J. and Hanski, C., Target genes of beta-catenin-T cell-factor/lymphoid-enhancer-factor signaling in human colorectal carcinomas. *Proc Natl Acad Sci U S A*, **96,** 1603-8 (1999).

Manousos, O., Day, N.E., Trichopoulos, D., Gerovassilis, F., Tzonou, A. and Polychronopoulou, A., Diet and colorectal cancer: a case-control study in Greece. *Int J Cancer*, **32,** 1-5 (1983).

Mariadason, J.M., Rickard, K.L., Barkla, D.H., Augenlicht, L.H. and Gibson, P.R., Divergent phenotypic patterns and commitment to apoptosis of Caco-2 cells during spontaneous and butyrate-induced differentiation. *J Cell Physiol*, **183,** 347-54 (2000).

Markowitz, S., Wang, J., Myeroff, L., Parsons, R., Sun, L., Lutterbaugh, J., Fan, R.S., Zborowska, E., Kinzler, K.W., Vogelstein, B. and et al., Inactivation of the type II TGF-beta receptor in colon cancer cells with microsatellite instability [see comments]. *Science*, **268,** 1336-8 (1995).

Marks, P.A., Richon, V.M. and Rifkind, R.A., Histone deacetylase inhibitors: inducers of differentiation or apoptosis of transformed cells. *J Natl Cancer Inst*, **92,** 1210-6 (2000).

Marnett, L.J., Aspirin and the potential role of prostaglandins in colon cancer. *Cancer Res*, **52,** 5575-89 (1992).

Martinez, M.E., Giovannucci, E.L., Colditz, G.A., Stampfer, M.J., Hunter, D.J., Speizer, F.E., Wing, A. and Willett, W.C., Calcium, vitamin D, and the occurrence of colorectal cancer among women. *J Natl Cancer Inst*, **88,** 1375-82 (1996).

Maurer, C.A., Friess, H., Buhler, S.S., Wahl, B.R., Graber, H., Zimmermann, A. and Buchler, M.W., Apoptosis inhibiting factor Bcl-xL might be the crucial member of the Bcl-2 gene family in colorectal cancer. *Dig Dis Sci*, **43,** 2641-8 (1998).

McIntyre, A., Gibson, P.R. and Young, G.P., Butyrate production from dietary fibre and protection against large bowel cancer in a rat model. *Gut*, **34,** 386-91 (1993).

Medema, R.H., Klompmaker, R., Smits, V.A. and Rijksen, G., p21waf1 can block cells at two points in the cell cycle, but does not interfere with processive DNA-replication or stress-activated kinases. *Oncogene*, **16,** 431-41 (1998).

Medina, V., Edmonds, B., Young, G.P., James, R., Appleton, S. and Zalewski, P.D., Induction of caspase-3 protease activity and apoptosis by butyrate and trichostatin A (inhibitors of histone deacetylase): dependence on protein synthesis and synergy with a mitochondrial/cytochrome c-dependent pathway. *Cancer Res*, **57,** 3697-707 (1997).

Melichar, B., Jandik, P., Malir, F., Tichy, M., Bures, J., Mergancova, J. and Voboril, Z., Increased urinary zinc and copper excretion in colorectal cancer. *J Trace Elem Electrolytes Health Dis*, **8,** 209-12 (1994).

Miller, T.L. and Wolin, M.J., Fermentations by saccharolytic intestinal bacteria. *Am J Clin Nutr*, **32,** 164-72 (1979).

Morana, S.J., Wolf, C.M., Li, J., Reynolds, J.E., Brown, M.K. and Eastman, A., The involvement of protein phosphatases in the activation of ICE/CED-3 protease, intracellular acidification, DNA digestion, and apoptosis. *J Biol Chem*, **271,** 18263-71 (1996).

Moss, S.F., Agarwal, B., Arber, N., Guan, R.J., Krajewska, M., Krajewski, S., Reed, J.C. and Holt, P.R., Increased intestinal Bak expression results in apoptosis. *Biochem Biophys Res Commun*, **223,** 199-203 (1996).

Moustakas, A. and Kardassis, D., Regulation of the human p21/WAF1/Cip1 promoter in hepatic cells by functional interactions between Sp1 and Smad family members. *Proc Natl Acad Sci U S A*, **95,** 6733-8 (1998).

Muir, J.G., Walker, K.Z., Kaimakamis, M.A., Cameron, M.A., Govers, M.J., Lu, Z.X., Young, G.P. and O'Dea, K., Modulation of fecal markers relevant to colon cancer risk: a high-starch Chinese diet did not generate expected beneficial changes relative to a Western-type diet. *Am J Clin Nutr*, **68,** 372-9 (1998).

Nagao, M., Ushijima, T., Wakabayashi, K., Ochiai, M., Kushida, H., Sugimura, T., Hasegawa, R., Shirai, T. and Ito, N., Dietary carcinogens and mammary carcinogenesis. Induction of rat mammary carcinomas by administration of heterocyclic amines in cooked foods. *Cancer*, **74,** 1063-9 (1994).

Nagengast, F.M., Grubben, M.J. and van Munster, I.P., Role of bile acids in colorectal carcinogenesis. *Eur J Cancer*, **31a,** 1067-70 (1995).

Nakano, K., Mizuno, T., Sowa, Y., Orita, T., Yoshino, T., Okuyama, Y., Fujita, T., Ohtani Fujita, N., Matsukawa, Y., Tokino, T., Yamagishi, H., Oka, T., Nomura, H. and Sakai, T.,

Butyrate activates the WAF1/Cip1 gene promoter through Sp1 sites in a p53-negative human colon cancer cell line. *J Biol Chem*, **272,** 22199-206 (1997).

Narisawa, T., Hermanek, P., Habs, M. and Schmahl, D., Reduction of carcinogenicity of N-nitrosomethylurea by indomethacin and failure of resuming effect of prostaglandin E2 (PGE2) against indomethacin. *J Cancer Res Clin Oncol*, **108,** 239-42 (1984).

Navert, B., Sandstrom, B. and Cederblad, A., Reduction of the phytate content of bran by leavening in bread and its effect on zinc absorption in man. *Br J Nutr*, **53,** 47-53 (1985).

Nelson, R.L., Abcarian, H., Nelson, T.M., Misumi, A., Kako, H., Rizk, S. and Sky Peck, H., The effect of dietary selenium deficiency on acute colorectal mucosal nucleotoxicity induced by several carcinogens in the rodent. *Am J Surg*, **172,** 85-8 (1996).

Nicholson, D.W., Ali, A., Thornberry, N.A., Vaillancourt, J.P., Ding, C.K., Gallant, M., Gareau, Y., Griffin, P.R., Labelle, M., Lazebnik, Y.A. and et al., Identification and inhibition of the ICE/CED-3 protease necessary for mammalian apoptosis [see comments]. *Nature*, **376,** 37-43 (1995).

Nicholson, D.W. and Thornberry, N.A., Caspases: killer proteases. *Trends Biochem Sci*, **22,** 299-306 (1997).

Nicoletti, I., Migliorati, G., Pagliacci, M.C., Grignani, F. and Riccardi, C., A rapid and simple method for measuring thymocyte apoptosis by propidium iodide staining and flow cytometry. *J Immunol Methods*, **139**, 271-9 (1991).

O'Connor, P., Mammalian G1 and G2 phase checkpoints. *In:* M.B. Kastan (ed.), *Checkpoint Controls and Cancer, Cancer Surveys*. Vol. 29, pp. 151-182, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY (1997).

O'Dell, B.L., Effect of dietary components upon zinc vailability. A review with original data. *Am J Clin Nutr*, **22,** 1315-22 (1969).

O'Halloran, T.V., Transition metals in control of gene expression [see comments]. *Science*, **261,** 715-25 (1993).

Okuno, S., Shimizu, S., Ito, T., Nomura, M., Hamada, E., Tsujimoto, Y. and Matsuda, H., Bcl-2 prevents caspase-independent cell death. *J Biol Chem*, **273,** 34272-7 (1998).

Oltvai, Z.N., Milliman, C.L. and Korsmeyer, S.J., Bcl-2 heterodimerizes in vivo with a conserved homolog, Bax, that accelerates programmed cell death. *Cell*, **74,** 609-19 (1993).

Oshima, M., Dinchuk, J.E., Kargman, S.L., Oshima, H., Hancock, B., Kwong, E., Trzaskos, J.M., Evans, J.F. and Taketo, M.M., Suppression of intestinal polyposis in Apc delta716 knockout mice by inhibition of cyclooxygenase 2 (COX-2). *Cell*, **87,** 803-9 (1996).

Owen, G.I., Richer, J.K., Tung, L., Takimoto, G. and Horwitz, K.B., Progesterone regulates transcription of the p21(WAF1) cyclin- dependent kinase inhibitor gene through Sp1 and CBP/p300. *J Biol Chem*, **273,** 10696-701 (1998).

Paganini Hill, A., Chao, A., Ross, R.K. and Henderson, B.E., Aspirin use and chronic diseases: a cohort study of the elderly [see comments]. *BMJ*, **299,** 1247-50 (1989).

Palmer, D.G., Paraskeva, C. and Williams, A.C., Modulation of p53 expression in cultured colonic adenoma cell lines by the naturally occurring lumenal factors butyrate and deoxycholate. *Int J Cancer*, **73,** 702-6 (1997).

Partik, G., Kahl Rainer, P., Sedivy, R., Ellinger, A., Bursch, W. and Marian, B., Apoptosis in human colorectal tumours: ultrastructure and quantitative studies on tissue localization and association with bak expression. *Virchows Arch*, **432,** 415-26 (1998).

Pecoud, A., Donzel, P. and Schelling, J.L., Effect of foodstuffs on the absorption of zinc sulfate. *Clin Pharmacol Ther*, **17,** 469-74 (1975).

Peeper, D.S., van der Eb, A.J. and Zantema, A., The G1/S cell-cycle checkpoint in eukaryotic cells. *Biochim Biophys Acta*, **1198,** 215-30 (1994).

Perry, D.K., Smyth, M.J., Stennicke, H.R., Salvesen, G.S., Duriez, P., Poirier, G.G. and Hannun, Y.A., Zinc is a potent inhibitor of the apoptotic protease, caspase-3. A novel target for zinc in the inhibition of apoptosis. *J Biol Chem*, **272,** 18530-3 (1997).

Phillips, J., Muir, J.G., Birkett, A., Lu, Z.X., Jones, G.P., O'Dea, K. and Young, G.P., Effect of resistant starch on fecal bulk and fermentation-dependent events in humans. *Am J Clin Nutr*, **62,** 121-30 (1995).

Piazza, G.A., Alberts, D.S., Hixson, L.J., Paranka, N.S., Li, H., Finn, T., Bogert, C., Guillen, J.M., Brendel, K., Gross, P.H., Sperl, G., Ritchie, J., Burt, R.W., Ellsworth, L., Ahnen, D.J. and Pamukcu, R., Sulindac sulfone inhibits azoxymethane-induced colon carcinogenesis in rats without reducing prostaglandin levels. *Cancer Res*, **57,** 2909-15 (1997).

Piazza, G.A., Rahm, A.L., Krutzsch, M., Sperl, G., Paranka, N.S., Gross, P.H., Brendel, K., Burt, R.W., Alberts, D.S., Pamukcu, R. and et al., Antineoplastic drugs sulindac sulfide and sulfone inhibit cell growth by inducing apoptosis. *Cancer Res*, **55,** 3110-6 (1995).

Poluha, W., Poluha, D.K., Chang, B., Crosbie, N.E., Schonhoff, C.M., Kilpatrick, D.L. and Ross, A.H., The cyclin-dependent kinase inhibitor p21 (WAF1) is required for survival of differentiating neuroblastoma cells. *Mol Cell Biol*, **16,** 1335-41 (1996).

Polyak, K., Waldman, T., He, T.C., Kinzler, K.W. and Vogelstein, B., Genetic determinants of p53-induced apoptosis and growth arrest. *Genes Dev*, **10,** 1945-52 (1996).

Potter, J.D., Nutrition and colorectal cancer. *Cancer Causes Control*, **7,** 127-46 (1996).

Prabhu, N.S., Blagosklonny, M.V., Zeng, Y.X., Wu, G.S., Waldman, T. and El Deiry, W.S., Suppression of cancer cell growth by adenovirus expressing p21(WAF1/CIP1) deficient in PCNA interaction. *Clin Cancer Res*, **2,** 1221-9 (1996).

Pretlow, T.P., O'Riordan, M.A., Somich, G.A., Amini, S.B. and Pretlow, T.G., Aberrant crypts correlate with tumor incidence in F344 rats treated with azoxymethane and phytate. *Carcinogenesis*, **13,** 1509-12 (1992).

Prowse, D.M., Bolgan, L., Molnar, A. and Dotto, G.P., Involvement of the Sp3 transcription factor in induction of p21Cip1/WAF1 in keratinocyte differentiation. *J Biol Chem*, **272,** 1308-14 (1997).

Rampino, N., Yamamoto, H., Ionov, Y., Li, Y., Sawai, H., Reed, J.C. and Perucho, M., Somatic frameshift mutations in the BAX gene in colon cancers of the microsatellite mutator phenotype. *Science*, **275,** 967-9 (1997).

Reddy, B.S., Prevention of colon carcinogenesis by components of dietary fiber. *Anticancer Res*, **19,** 3681-3 (1999).

Reddy, B.S., Maeura, Y. and Wayman, M., Effect of dietary corn bran and autohydrolyzed lignin on 3,2'-dimethyl-4-aminobiphenyl-induced intestinal carcinogenesis in male F344 rats. *J Natl Cancer Inst*, **71,** 419-23 (1983).

Reddy, B.S., Maruyama, H. and Kelloff, G., Dose-related inhibition of colon carcinogenesis by dietary piroxicam, a nonsteroidal antiinflammatory drug, during different stages of rat colon tumor development. *Cancer Res*, **47,** 5340-6 (1987).

Reddy, B.S. and Mori, H., Effect of dietary wheat bran and dehydrated citrus fiber on 3,2'-dimethyl-4-aminobiphenyl-induced intestinal carcinogenesis in F344 rats. *Carcinogenesis*, **2,** 21-5 (1981).

Reddy, B.S., Mori, H. and Nicolais, M., Effect of dietary wheat bran and dehydrated citrus fiber on azoxymethane-induced intestinal carcinogenesis in Fischer 344 rats. *J Natl Cancer Inst*, **66,** 553-7 (1981).

Reddy, B.S., Rivenson, A., El Bayoumy, K., Upadhyaya, P., Pittman, B. and Rao, C.V., Chemoprevention of colon cancer by organoselenium compounds and impact of high- or low-fat diets. *J Natl Cancer Inst*, **89,** 506-12 (1997).

Roberts Thomson, I.C., Ryan, P., Khoo, K.K., Hart, W.J., McMichael, A.J. and Butler, R.N., Diet, acetylator phenotype, and risk of colorectal neoplasia. *Lancet*, **347,** 1372-4 (1996).

Rodrigues, N.R., Rowan, A., Smith, M.E., Kerr, I.B., Bodmer, W.F., Gannon, J.V. and Lane, D.P., p53 mutations in colorectal cancer. *Proc Natl Acad Sci U S A*, **87,** 7555-9 (1990).

Roediger, W.E., Utilization of nutrients by isolated epithelial cells of the rat colon. *Gastroenterology*, **83,** 424-9 (1982).

Roger, R., Issaad, C., Pallardy, M., Leglise, M.C., Turhan, A.G., Bertoglio, J. and Breard, J., BCR-ABL does not prevent apoptotic death induced by human natural killer or lymphokine-activated killer cells. *Blood*, **87**, 1113-22 (1996).

Rubinfeld, B., Albert, I., Porfiri, E., Fiol, C., Munemitsu, S. and Polakis, P., Binding of GSK3beta to the APC-beta-catenin complex and regulation of complex assembly [see comments]. *Science*, **272,** 1023-6 (1996).

Ruschoff, J., Wallinger, S., Dietmaier, W., Bocker, T., Brockhoff, G., Hofstadter, F. and Fishel, R., Aspirin suppresses the mutator phenotype associated with hereditary nonpolyposis colorectal cancer by genetic selection. *Proc Natl Acad Sci U S A*, **95,** 11301-6 (1998).

Saied, I.T. and Shamsuddin, A.M., Up-regulation of the tumor suppressor gene p53 and WAF1 gene expression by IP6 in HT-29 human colon carcinoma cell line. *Anticancer Res*, **18,** 1479-84 (1998).

Saito, H., Ebinuma, H., Takahashi, M., Kaneko, F., Wakabayashi, K., Nakamura, M. and Ishii, H., Loss of butyrate-induced apoptosis in human hepatoma cell lines HCC-M and HCC-T having substantial Bcl-2 expression. *Hepatology*, **27,** 1233-40 (1998).

Sakahira, H., Enari, M. and Nagata, S., Cleavage of CAD inhibitor in CAD activation and DNA degradation during apoptosis [see comments]. *Nature*, **391,** 96-9 (1998).

Sakamoto, K., Venkatraman, G. and Shamsuddin, A.M., Growth inhibition and differentiation of HT-29 cells in vitro by inositol hexaphosphate (phytic acid). *Carcinogenesis*, **14,** 1815-9 (1993).

Sakamoto, K., Vucenik, I. and Shamsuddin, A.M., [3H]phytic acid (inositol hexaphosphate) is absorbed and distributed to various tissues in rats. *J Nutr*, **123,** 713-20 (1993).

Salahshor, S., Kressner, U., Pahlman, L., Glimelius, B., Lindmark, G. and Lindblom, A., Colorectal cancer with and without microsatellite instability involves different genes. *Genes Chromosomes Cancer*, **26,** 247-52 (1999).

Sandberg, A.S. and Ahderinne, R., HPLC method for determination of inositol tri-, tetra-, penta-, and hexaphosphates in foods and intestinal contents. *J Food Sci*, **51,** 547-550 (1986).

Sandstrom, B., Cederblad, A. and Lonnerdal, B., Zinc absorption from human milk, cow's milk, and infant formulas. *Am J Dis Child*, **137,** 726-9 (1983).

Sano, H., Kawahito, Y., Wilder, R.L., Hashiramoto, A., Mukai, S., Asai, K., Kimura, S., Kato, H., Kondo, M. and Hla, T., Expression of cyclooxygenase-1 and -2 in human colorectal cancer. *Cancer Res*, **55,** 3785-9 (1995).

Schatzkin, A., Lanza, E., Corle, D., Lance, P., Iber, F., Caan, B., Shike, M., Weissfeld, J., Burt, R., Cooper, M.R., Kikendall, J.W. and Cahill, J., Lack of effect of a low-fat, high-fiber diet on the recurrence of colorectal adenomas. Polyp Prevention Trial Study Group [see comments]. *N Engl J Med*, **342,** 1149-55 (2000).

Schott, A.F., Apel, I.J., Nunez, G. and Clarke, M.F., Bcl-XL protects cancer cells from p53-mediated apoptosis. *Oncogene*, **11,** 1389-94 (1995).

Schwartz, S., Jr., Yamamoto, H., Navarro, M., Maestro, M., Reventos, J. and Perucho, M., Frameshift mutations at mononucleotide repeats in caspase-5 and other target genes in endometrial and gastrointestinal cancer of the microsatellite mutator phenotype. *Cancer Res*, **59,** 2995-3002 (1999).

Shaham, S. and Horvitz, H.R., Developing Caenorhabditis elegans neurons may contain both cell-death protective and killer activities. *Genes Dev*, **10,** 578-91 (1996).

Shamsuddin, A.M., Inositol phosphates have novel anticancer function. *J Nutr*, **125,** 725S-732S (1995).

Shamsuddin, A.M., Metabolism and cellular functions of IP6: a review. *Anticancer Res*, **19,** 3733-6 (1999).

Shamsuddin, A.M., Baten, A. and Lalwani, N.D., Effects of inositol hexaphosphate on growth and differentiation in K-562 erythroleukemia cell line. *Cancer Lett*, **64,** 195-202 (1992).

Shamsuddin, A.M., Elsayed, A.M. and Ullah, A., Suppression of large intestinal cancer in F344 rats by inositol hexaphosphate. *Carcinogenesis*, **9,** 577-80 (1988).

Shamsuddin, A.M. and Ullah, A., Inositol hexaphosphate inhibits large intestinal cancer in F344 rats 5 months after induction by azoxymethane. *Carcinogenesis*, **10,** 625-6 (1989).

Shamsuddin, A.M., Ullah, A. and Chakravarthy, A.K., Inositol and inositol hexaphosphate suppress cell proliferation and tumor formation in CD-1 mice. *Carcinogenesis*, **10,** 1461-3 (1989).

Shamsuddin, A.M., Vucenik, I. and Cole, K.E., IP6: a novel anti-cancer agent. *Life Sci*, **61,** 343-54 (1997).

Shamsuddin, A.M. and Yang, G.Y., Inositol hexaphosphate inhibits growth and induces differentiation of PC-3 human prostate cancer cells. *Carcinogenesis*, **16,** 1975-9 (1995).

Shamsuddin, A.M., Yang, G.Y. and Vucenik, I., Novel anti-cancer functions of IP6: growth inhibition and differentiation of human mammary cancer cell lines in vitro. *Anticancer Res*, **16,** 3287-92 (1996).

Sheikh, M.S., Chen, Y.Q., Smith, M.L. and Fornace, A.J., Jr., Role of p21Waf1/Cip1/Sdi1 in cell death and DNA repair as studied using a tetracycline-inducible system in p53-deficient cells. *Oncogene*, **14,** 1875-82 (1997).

Sheikh, M.S., Rochefort, H. and Garcia, M., Overexpression of p21WAF1/CIP1 induces growth arrest, giant cell formation and apoptosis in human breast carcinoma cell lines. *Oncogene*, **11,** 1899-905 (1995).

Shiff, S.J., Qiao, L., Tsai, L.L. and Rigas, B., Sulindac sulfide, an aspirin-like compound, inhibits proliferation, causes cell cycle quiescence, and induces apoptosis in HT-29 colon adenocarcinoma cells. *J Clin Invest*, **96,** 491-503 (1995).

Shike, M., Winawer, S.J., Greenwald, P.H., Bloch, A., Hill, M.J. and Swaroop, S.V., Primary prevention of colorectal cancer. The WHO Collaborating Centre for the Prevention of Colorectal Cancer. *Bull World Health Organ*, **68,** 377-85 (1990).

Shiohara, M., Koike, K., Komiyama, A. and Koeffler, H.P., p21WAF1 mutations and human malignancies. *Leuk Lymphoma*, **26,** 35-41 (1997).

Shivapurkar, N., Tang, Z.C., Frost, A. and Alabaster, O., A rapid dual organ rat carcinogenesis bioassay for evaluating the chemoprevention of breast and colon cancer. *Cancer Lett*, **100,** 169-79 (1996).

Shivji, M.K., Grey, S.J., Strausfeld, U.P., Wood, R.D. and Blow, J.J., Cip1 inhibits DNA replication but not PCNA-dependent nucleotide excision-repair. *Curr Biol*, **4,** 1062-8 (1994).

Siavoshian, S., Blottiere, H.M., Cherbut, C. and Galmiche, J.P., Butyrate stimulates cyclin D and p21 and inhibits cyclin-dependent kinase 2 expression in HT-29 colonic epithelial cells. *Biochem Biophys Res Commun*, **232,** 169-72 (1997).

Siavoshian, S., Segain, J.P., Kornprobst, M., Bonnet, C., Cherbut, C., Galmiche, J.P. and Blottiere, H.M., Butyrate and trichostatin A effects on the proliferation/differentiation of human intestinal epithelial cells: induction of cyclin D3 and p21 expression. *Gut*, **46,** 507-14 (2000).

Singh, B. and Paraskeva, C., Bcl-2 as a possible sensor of nutritional stress inhibiting apoptosis and allowing cell survival during colorectal carcinogenesis. *Biochem Soc Trans*, **26,** 236-41 (1998).

Sinicrope, F.A., Roddey, G., Lemoine, M., Ruan, S., Stephens, L.C., Frazier, M.L., Shen, Y. and Zhang, W., Loss of p21WAF1/Cip1 protein expression accompanies progression of sporadic colorectal neoplasms but not hereditary nonpolyposis colorectal cancers. *Clin Cancer Res*, **4,** 1251-61 (1998).

Sinicrope, F.A., Roddey, G., McDonnell, T.J., Shen, Y., Cleary, K.R. and Stephens, L.C., Increased apoptosis accompanies neoplastic development in the human colorectum. *Clin Cancer Res*, **2,** 1999-2006 (1996).

Sitrin, M.D., Halline, A.G., Abrahams, C. and Brasitus, T.A., Dietary calcium and vitamin D modulate 1,2-dimethylhydrazine-induced colonic carcinogenesis in the rat. *Cancer Res*, **51,** 5608-13 (1991).

Sjolander, A., Schippert, A. and Hammarstrom, S., A human epithelial cell line, intestine 407, can produce 5-hydroxyeicosatetraenoic acid and leukotriene B4. *Prostaglandins*, **45,** 85-96 (1993).

Slee, E.A., Harte, M.T., Kluck, R.M., Wolf, B.B., Casiano, C.A., Newmeyer, D.D., Wang, H.G., Reed, J.C., Nicholson, D.W., Alnemri, E.S., Green, D.R. and Martin, S.J., Ordering the cytochrome c-initiated caspase cascade: hierarchical activation of caspases-2, -3, -6, -7, -8, and -10 in a caspase-9-dependent manner. *J Cell Biol*, **144,** 281-92 (1999).

Smith, M.L., Hawcroft, G. and Hull, M.A., The effect of non-steroidal anti-inflammatory drugs on human colorectal cancer cells: evidence of different mechanisms of action. *Eur J Cancer*, **36,** 664-74 (2000).

Solomons, N.W., Zinc and copper. *In:* M.E. Shils and V.R. Young (eds.), *Modern Nutrition in health and Disease, 7th*, pp. 238-762, Lea Febiger, Philadephia (1988).

Song, M.K., Adham, N.F., Heng, M.C., Costea, N.V., Heng, M.K. and Ament, M.E., Metabolic alterations of zinc and prostaglandins in both human and animal colonic tumor cells. *J Am Coll Nutr*, **14,** 473-9 (1995).

Song, M.K., Heng, M.C., Rolandelli, R., Ament, M.E. and Heng, M.K., Possible link between zinc intake and colon cancer. *J Natl Cancer Inst*, **85,** 667-9 (1993).

Sowa, Y., Orita, T., Minamikawa Hiranabe, S., Mizuno, T., Nomura, H. and Sakai, T., Sp3, but not Sp1, mediates the transcriptional activation of the p21/WAF1/Cip1 gene promoter by histone deacetylase inhibitor. *Cancer Res*, **59,** 4266-70 (1999).

Srinivasula, S.M., Fernandes Alnemri, T., Zangrilli, J., Robertson, N., Armstrong, R.C., Wang, L., Trapani, J.A., Tomaselli, K.J., Litwack, G. and Alnemri, E.S., The Ced-3/interleukin 1beta converting enzyme-like homolog Mch6 and the lamin-cleaving enzyme Mch2alpha are substrates for the apoptotic mediator CPP32. *J Biol Chem*, **271,** 27099-106 (1996).

Steele, V.E., Holmes, C.A., Hawk, E.T., Kopelovich, L., Lubet, R.A., Crowell, J.A., Sigman, C.C. and Kelloff, G.J., Lipoxygenase inhibitors as potential cancer chemopreventives. *Cancer Epidemiol Biomarkers Prev*, **8,** 467-83 (1999).

Steinbach, G., Lynch, P.M., Phillips, R.K., Wallace, M.H., Hawk, E., Gordon, G.B., Wakabayashi, N., Saunders, B., Shen, Y., Fujimura, T., Su, L.K. and Levin, B., The effect of celecoxib, a cyclooxygenase-2 inhibitor, in familial adenomatous polyposis. *N Engl J Med*, **342,** 1946-52 (2000).

Stennicke, H.R. and Salvesen, G.S., Biochemical characteristics of caspases-3, -6, -7, and -8. *J Biol Chem*, **272,** 25719-23 (1997).

Susin, S.A., Lorenzo, H.K., Zamzami, N., Marzo, I., Snow, B.E., Brothers, G.M., Mangion, J., Jacotot, E., Costantini, P., Loeffler, M., Larochette, N., Goodlett, D.R., Aebersold, R., Siderovski, D.P., Penninger, J.M. and Kroemer, G., Molecular characterization of mitochondrial apoptosis-inducing factor [see comments]. *Nature*, **397,** 441-6 (1999).

Suzuki, A., Tsutomi, Y., Akahane, K., Araki, T. and Miura, M., Resistance to Fas-mediated apoptosis: activation of caspase 3 is regulated by cell cycle regulator p21WAF1 and IAP gene family ILP. *Oncogene*, **17,** 931-9 (1998).

Takahashi, A., Alnemri, E.S., Lazebnik, Y.A., Fernandes Alnemri, T., Litwack, G., Moir, R.D., Goldman, R.D., Poirier, G.G., Kaufmann, S.H. and Earnshaw, W.C., Cleavage of lamin A by Mch2 alpha but not CPP32: multiple interleukin 1 beta-converting enzyme-related proteases with distinct substrate recognition properties are active in apoptosis. *Proc Natl Acad Sci U S A*, **93,** 8395-400 (1996).

Tanaka, T., Makita, H., Kawabata, K., Mori, H., Kakumoto, M., Satoh, K., Hara, A., Sumida, T. and Ogawa, H., Chemoprevention of azoxymethane-induced rat colon carcinogenesis by the naturally occurring flavonoids, diosmin and hesperidin. *Carcinogenesis*, **18,** 957-65 (1997).

Tang, D. and Kidd, V.J., Cleavage of DFF-45/ICAD by multiple caspases is essential for its function during apoptosis. *J Biol Chem*, **273,** 28549-52 (1998).

Tang, D.G., Chen, Y.Q. and Honn, K.V., Arachidonate lipoxygenases as essential regulators of cell survival and apoptosis. *Proc Natl Acad Sci U S A*, **93,** 5241-6 (1996).

Tang, D.G. and Honn, K.V., 12-Lipoxygenase, 12(S)-HETE, and cancer metastasis. *Ann N Y Acad Sci*, **744,** 199-215 (1994).

Teare, J.M., Islam, R., Flanagan, R., Gallagher, S., Davies, M.G. and Grabau, C., Measurement of nucleic acid concentrations using the DyNA Quant and the GeneQuant. *Biotechniques*, **22,** 1170-4 (1997).

Tetsu, O. and McCormick, F., Beta-catenin regulates expression of cyclin D1 in colon carcinoma cells. *Nature*, **398,** 422-6 (1999).

Thompson, H.J., Jiang, C., Lu, J., Mehta, R.G., Piazza, G.A., Paranka, N.S., Pamukcu, R. and Ahnen, D.J., Sulfone metabolite of sulindac inhibits mammary carcinogenesis. *Cancer Res*, **57,** 267-71 (1997).

Thornberry, N.A. and Lazebnik, Y., Caspases: enemies within. *Science*, **281,** 1312-6 (1998).

Thornberry, N.A., Rano, T.A., Peterson, E.P., Rasper, D.M., Timkey, T., Garcia Calvo, M., Houtzager, V.M., Nordstrom, P.A., Roy, S., Vaillancourt, J.P., Chapman, K.T. and Nicholson, D.W., A combinatorial approach defines specificities of members of the caspase family and granzyme B. Functional relationships established for key mediators of apoptosis. *J Biol Chem*, **272,** 17907-11 (1997).

Thorson, A.G., Knezetic, J.A. and Lynch, H.T., A century of progress in hereditary nonpolyposis colorectal cancer (Lynch syndrome). *Dis Colon Rectum*, **42,** 1-9 (1999).

Thun, M.J., Calle, E.E., Namboodiri, M.M., Flanders, W.D., Coates, R.J., Byers, T., Boffetta, P., Garfinkel, L. and Heath, C.W., Jr., Risk factors for fatal colon cancer in a large prospective study [see comments]. *J Natl Cancer Inst*, **84,** 1491-500 (1992).

Thun, M.J., Namboodiri, M.M. and Heath, C.W., Jr., Aspirin use and reduced risk of fatal colon cancer [see comments]. *N Engl J Med*, **325,** 1593-6 (1991).

Tian, H., Wittmack, E.K. and Jorgensen, T.J., p21WAF1/CIP1 antisense therapy radiosensitizes human colon cancer by converting growth arrest to apoptosis. *Cancer Res*, **60,** 679-84 (2000).

Timar, J., Raso, E., Fazakas, Z.S., Silletti, S., Raz, A. and Honn, K.V., Multiple use of a signal transduction pathway in tumor cell invasion. *Anticancer Res*, **16,** 3299-306 (1996).

Trock, B., Lanza, E. and Greenwald, P., Dietary fiber, vegetables, and colon cancer: critical review and meta-analyses of the epidemiologic evidence. *J Natl Cancer Inst*, **82,** 650-61 (1990).

Tsujii, M. and DuBois, R.N., Alterations in cellular adhesion and apoptosis in epithelial cells overexpressing prostaglandin endoperoxide synthase 2. *Cell*, **83,** 493-501 (1995).

Tsujimoto, Y., Role of Bcl-2 family proteins in apoptosis: apoptosomes or mitochondria? *Genes Cells*, **3,** 697-707 (1998).

Tsujimoto, Y., Cossman, J., Jaffe, E. and Croce, C.M., Involvement of the bcl-2 gene in human follicular lymphoma. *Science*, **228,** 1440-3 (1985).

Tsujitani, S., Shirai, H., Tatebe, S., Sugamura, K., Ohfuji, S., Gomyo, Y., Maeta, M., Ito, H. and Kaibara, N., Apoptotic cell death and its relationship to carcinogenesis in colorectal carcinoma. *Cancer*, **77,** 1711-6 (1996).

Ullah, A. and Shamsuddin, A.M., Dose-dependent inhibition of large intestinal cancer by inositol hexaphosphate in F344 rats. *Carcinogenesis*, **11,** 2219-22 (1990).

Umar, A., Buermeyer, A.B., Simon, J.A., Thomas, D.C., Clark, A.B., Liskay, R.M. and Kunkel, T.A., Requirement for PCNA in DNA mismatch repair at a step preceding DNA resynthesis. *Cell*, **87,** 65-73 (1996).

Urbano, A., McCaffrey, R. and Foss, F., Isolation and characterization of NUC70, a cytoplasmic, hematopoietic apoptotic endonuclease. *J Biol Chem*, **273,** 34820-7 (1998).

Vallee, B.L. and Falchuk, K.H., The biochemical basis of zinc physiology. *Physiol Rev*, **73,** 79-118 (1993).

Vaziri, C., Stice, L. and Faller, D.V., Butyrate-induced G1 arrest results from p21-independent disruption of retinoblastoma protein-mediated signals. *Cell Growth Differ*, **9,** 465-74 (1998).

Vento, R., D'Alessandro, N., Giuliano, M., Lauricella, M., Carabillo, M. and Tesoriere, G., Induction of apoptosis by arachidonic acid in human retinoblastoma Y79 cells: involvement of oxidative stress. *Exp Eye Res*, **70,** 503-17 (2000).

Vermes, I., Haanen, C., Steffens Nakken, H. and Reutelingsperger, C., A novel assay for apoptosis. Flow cytometric detection of phosphatidylserine expression on early apoptotic cells using fluorescein labelled Annexin V. *J Immunol Methods*, **184**, 39-51 (1995).

Vettese Dadey, M., Grant, P.A., Hebbes, T.R., Crane Robinson, C., Allis, C.D. and Workman, J.L., Acetylation of histone H4 plays a primary role in enhancing transcription factor binding to nucleosomal DNA in vitro. *EMBO J*, **15,** 2508-18 (1996).

Vohra, P., Gray, G.A. and Kratzer, F.H., Phytic acid-metal complexes. *Proc Soc Exp Biol Med*, **120,** 447-9 (1965).

Vohra, P. and Kratzer, F.H., Influence of various phosphates and other complexing agents on the availability of zinc for turkey poults. *J Nutr*, **89,** 106-12 (1966).

Vucenik, I., Sakamoto, K., Bansal, M. and Shamsuddin, A.M., Inhibition of rat mammary carcinogenesis by inositol hexaphosphate (phytic acid). A pilot study. *Cancer Lett*, **75,** 95-102 (1993).

Vucenik, I. and Shamsuddin, A.M., [3H]inositol hexaphosphate (phytic acid) is rapidly absorbed and metabolized by murine and human malignant cells in vitro. *J Nutr*, **124,** 861-8 (1994).

Vucenik, I., Yang, G.Y. and Shamsuddin, A.M., Inositol hexaphosphate and inositol inhibit DMBA-induced rat mammary cancer. *Carcinogenesis*, **16,** 1055-8 (1995).

Vucenik, I., Zhang, Z.S. and Shamsuddin, A.M., IP6 in treatment of liver cancer. II. Intra-tumoral injection of IP6 regresses pre-existing human liver cancer xenotransplanted in nude mice. *Anticancer Res*, **18,** 4091-6 (1998).

Waddell, W.R., Ganser, G.F., Cerise, E.J. and Loughry, R.W., Sulindac for polyposis of the colon. *Am J Surg*, **157,** 175-9 (1989).

Waddell, W.R. and Loughry, R.W., Sulindac for polyposis of the colon. *J Surg Oncol*, **24,** 83-7 (1983).

Waga, S., Hannon, G.J., Beach, D. and Stillman, B., The p21 inhibitor of cyclin-dependent kinases controls DNA replication by interaction with PCNA [see comments]. *Nature*, **369,** 574-8 (1994).

Waga, S. and Stillman, B., Cyclin-dependent kinase inhibitor p21 modulates the DNA primer-template recognition complex. *Mol Cell Biol*, **18,** 4177-87 (1998).

Waldman, T., Zhang, Y., Dillehay, L., Yu, J., Kinzler, K., Vogelstein, B. and Williams, J., Cell-cycle arrest versus cell death in cancer therapy [see comments]. *Nat Med*, **3,** 1034-6 (1997).

Wang, H., Grand, R.J., Milner, A.E., Armitage, R.J., Gordon, J. and Gregory, C.D., Repression of apoptosis in human B-lymphoma cells by CD40-ligand and Bcl-2: relationship to the cell-cycle and role of the retinoblastoma protein. *Oncogene*, **13,** 373-9 (1996).

Wargovich, M.J., Eng, V.W., Newmark, H.L. and Bruce, W.R., Calcium ameliorates the toxic effect of deoxycholic acid on colonic epithelium. *Carcinogenesis*, **4,** 1205-7 (1983).

Wechter, W.J., Kantoci, D., Murray, E.D., Jr., Quiggle, D.D., Leipold, D.D., Gibson, K.M. and McCracken, J.D., R-flurbiprofen chemoprevention and treatment of intestinal adenomas in the APC(Min)/+ mouse model: implications for prophylaxis and treatment of colon cancer. *Cancer Res*, **57,** 4316-24 (1997).

Weinberg, R.A., The retinoblastoma protein and cell cycle control. *Cell*, **81,** 323-30 (1995).

Weisburger, J.H., Rivenson, A., Aliaga, C., Reinhardt, J., Kelloff, G.J., Boone, C.W., Steele, V.E., Balentine, D.A., Pittman, B. and Zang, E., Effect of tea extracts, polyphenols, and epigallocatechin gallate on azoxymethane-induced colon cancer. *Proc Soc Exp Biol Med*, **217,** 104-8 (1998).

Widmann, C., Gibson, S. and Johnson, G.L., Caspase-dependent cleavage of signaling proteins during apoptosis. A turn-off mechanism for anti-apoptotic signals. *J Biol Chem*, **273,** 7141-7 (1998).

Wijnen, J.T., Vasen, H.F., Khan, P.M., Zwinderman, A.H., van der Klift, H., Mulder, A., Tops, C., Moller, P. and Fodde, R., Clinical findings with implications for genetic testing in families with clustering of colorectal cancer. *N Engl J Med*, **339,** 511-8 (1998).

Willett, W.C., Stampfer, M.J., Colditz, G.A., Rosner, B.A. and Speizer, F.E., Relation of meat, fat, and fiber intake to the risk of colon cancer in a prospective study among women [see comments]. *N Engl J Med*, **323,** 1664-72 (1990).

Winde, G., Gumbinger, H.G., Osswald, H., Kemper, F. and Bunte, H., The NSAID sulindac reverses rectal adenomas in colectomized patients with familial adenomatous polyposis: clinical results of a dose-finding study on rectal sulindac administration. *Int J Colorectal Dis*, **8,** 13-7 (1993).

Winde, G., Schmid, K.W., Schlegel, W., Fischer, R., Osswald, H. and Bunte, H., Complete reversion and prevention of rectal adenomas in colectomized patients with familial adenomatous polyposis by rectal low-dose sulindac maintenance treatment. Advantages of a low-dose nonsteroidal anti-inflammatory drug regimen in reversing adenomas exceeding 33 months. *Dis Colon Rectum*, **38,** 813-30 (1995).

Wintersberger, E., Mudrak, I. and Wintersberger, U., Butyrate inhibits mouse fibroblasts at a control point in the G1 phase. *J Cell Biochem*, **21,** 239-47 (1983).

Wolf, C.M. and Eastman, A., The temporal relationship between protein phosphatase, mitochondrial cytochrome c release, and caspase activation in apoptosis. *Exp Cell Res*, **247,** 505-13 (1999).

World-Cancer-Research-Fund, Food, Nutrition and the Prevention of Cancer, American Institute for Cancer Research, Washington DC (1997).

Wyllie, A.H., Glucocorticoid-induced thymocyte apoptosis is associated with endogenous endonuclease activation. *Nature*, **284,** 555-6 (1980).

Wyllie, A.H., Kerr, J.F. and Currie, A.R., Cell death: the significance of apoptosis. *Int Rev Cytol*, **68**, 251-306 (1980).

Xiao, H., Hasegawa, T., Miyaishi, O., Ohkusu, K. and Isobe, K., Sodium butyrate induces NIH3T3 cells to senescence-like state and enhances promoter activity of p21WAF/CIP1 in p53-independent manner. *Biochem Biophys Res Commun*, **237,** 457-60 (1997).

Xiao, K.Q. and Henderson, W.J., [Electron microscopy microanalysis and quantitative detection of trace elements in carcinoma of the colon]. *Chung Hua Ping Li Hsueh Tsa Chih*, **21,** 142-5 (1992).

Xiong, Y., Hannon, G.J., Zhang, H., Casso, D., Kobayashi, R. and Beach, D., p21 is a universal inhibitor of cyclin kinases [see comments]. *Nature*, **366,** 701-4 (1993).

Yagi, O.K., Akiyama, Y., Nomizu, T., Iwama, T., Endo, M. and Yuasa, Y., Proapoptotic gene BAX is frequently mutated in hereditary nonpolyposis colorectal cancers but not in adenomas. *Gastroenterology*, **114,** 268-74 (1998).

Yamada, K., Ohtsu, M., Sugano, M. and Kimura, G., Effects of butyrate on cell cycle progression and polyploidization of various types of mammalian cells. *Biosci Biotechnol Biochem*, **56,** 1261-5 (1992).

Yamamoto, H., Fujimoto, J., Okamoto, E., Furuyama, J., Tamaoki, T. and Hashimoto Tamaoki, T., Suppression of growth of hepatocellular carcinoma by sodium butyrate in vitro and in vivo. *Int J Cancer*, **76,** 897-902 (1998).

Yang, E., Zha, J., Jockel, J., Boise, L.H., Thompson, C.B. and Korsmeyer, S.J., Bad, a heterodimeric partner for Bcl-XL and Bcl-2, displaces Bax and promotes cell death. *Cell*, **80,** 285-91 (1995).

Yang, G.Y. and Shamsuddin, A.M., IP6-induced growth inhibition and differentiation of HT-29 human colon cancer cells: involvement of intracellular inositol phosphates. *Anticancer Res*, **15,** 2479-87 (1995).

Yang, H.B., Chow, N.H., Sheu, B.S., Chan, S.H., Chien, C.H. and Su, I.J., The role of bcl-2 in the progression of the colorectal adenoma-carcinoma sequence. *Anticancer Res*, **19,** 727-30 (1999).

Yen, A. and Sturgill, R., Hypophosphorylation of the RB protein in S and G2 as well as G1 during growth arrest. *Exp Cell Res*, **241,** 324-31 (1998).

Yoshitaka, T., Matsubara, N., Ikeda, M., Tanino, M., Hanafusa, H., Tanaka, N. and Shimizu, K., Mutations of E2F-4 trinucleotide repeats in colorectal cancer with microsatellite instability. *Biochem Biophys Res Commun*, **227,** 553-7 (1996).

Young, G.P., Butyrate and the colorectal cancer cells. *In:* H.J. Binder, J.H. Cummings and K. Soergel (eds.), *Short Chain Fatty Acids*, pp. 148-160, Kluwer, Lancaster, UK (1994).

Young, G.P., Contrasting effects of butyrate on proliferation and differentiation of normal and neoplastic cells. *In:* J.L. Rombeau, J.H. Cummings and T. Sakata (eds.), *Short Chain Fatty Acids: Metabolism and Clinical Importance*, pp. 50-55, Ross Laboratories, Columbus, OH (1991).

Young, G.P., Screening for colorectal cancer: an introduction. *In:* G.P. Young, B. Levin and P. Rozen (eds.), *Prevention and Early Detection of Colorectal Cancer*, pp. 271-274, WB Saunders, London, UK (1996).

Young, G.P., Chai, F. and Zalewski, P.D., Primary prevention of colorectal cancer. *In:* S.R. Li (ed.), *Colorectal Cancer: Early Diagnosis, Therapy and Prevention*, pp. 365-380, Science Publishing House, Beijing (2000).

Young, G.P., McIntyre, A., Albert, V., Folino, M., Muir, J.G. and Gibson, P.R., Wheat bran suppresses potato starch--potentiated colorectal tumorigenesis at the aberrant crypt stage in a rat model. *Gastroenterology*, **110,** 508-14 (1996).

Young, T.B. and Wolf, D.A., Case-control study of proximal and distal colon cancer and diet in Wisconsin. *Int J Cancer*, **42,** 167-75 (1988).

Zalewski, p.D. and Forbes, I.J., Intracellular zinc and the regulation of apoptosis. *In:* M. Lavin and D. Watters (eds.), *Programmed Cell Death: The Cellular and Molecular Biology of Apoptosis.*, pp. 73-86, Harwood Academic Publishers, Melbourne (1993).

Zalewski, P.D., Forbes, I.J. and Betts, W.H., Correlation of apoptosis with change in intracellular labile Zn(II) using zinquin [(2-methyl-8-p-toluenesulphonamido-6-quinolyloxy)acetic acid], a new specific fluorescent probe for Zn(II). *Biochem J*, **296,** 403-8 (1993).

Zamzami, N., Marchetti, P., Castedo, M., Zanin, C., Vayssiere, J.L., Petit, P.X. and Kroemer, G., Reduction in mitochondrial potential constitutes an early irreversible step of programmed lymphocyte death in vivo. *J Exp Med*, **181**, 1661-72 (1995).

Zhang, X., Morham, S.G., Langenbach, R. and Young, D.A., Malignant transformation and antineoplastic actions of nonsteroidal antiinflammatory drugs (NSAIDs) on cyclooxygenase-null embryo fibroblasts [see comments]. *J Exp Med*, **190,** 451-59 (1999).

Zhang, Y., Fujita, N. and Tsuruo, T., Caspase-mediated cleavage of p21Waf1/Cip1 converts cancer cells from growth arrest to undergoing apoptosis. *Oncogene*, **18,** 1131-8 (1999).

PUBLISHED MANUSCRIPTS RELEVANT TO THIS THESIS

Chai, F., Truong-Tran, A.Q., Ho, L.H. & Zalewski, P.D. (1999) Regulation of caspase activation and apoptosis by cellular zinc fluxes and zinc deprivation: a review.
Immunology and Cell Biology, v. 77, pp. 272-278

Young, G.P., Chai, F. & Zalewski, P. (1999) Polysaccaride fermentation, butyrate and apoptosis in the colonic epithelium.
Asia Pacific Journal of Clinical Nutrition, v. 8(suppl), pp. S27-31

Chai, F., Evdokiou, A., Young, G.P. & Zalewski, P.D. (2000) Involvement of p21 Waf1/Cip1 and its cleavage by DEVD-caspase during apoptosis of colorectal cancer cells induced by butyrate. *Carcinogenesis, v. 21(1), pp. 7-14*

Chai, F., Truong-Tran, A.Q., Ezdokiou, A., Young, G.P. & Zalewski, P.D. (2000) Intracellular zinc depletion induces caspase activation and p21 Waf1/Cip1 cleavage in human epithelial cell lines. *Journal of Infectious Diseses, v. 182(suppl), pp. S85-92*

Truong-Tran, A.Q., Ho, L.H., Chai, F. & Zalewski, P.D. (2000) Cellular zinc fluxes and the regulation of apoptosis/gene-directed cell death.
Journal of Nutrition, v. 130(5), pp. 1459S-1466S

LIST OF MINOR CORRECTIONS

Page	Line	Correction
3	9	Change "k-Ras" to "Ki-Ras"
4	15	Change "are unlikely to be" to "may not be"
16	17	Insert after " by apoptosis" "(Shanmugathasan and Jothy, 2000)"
19	15	Insert after ".... polyps" "(Fearon and Vogelstein, 1990)."
19	17	Insert after ".... cancers" "(Baker *et al.*, 1989)."
33	8-9	Change ".... converted cell growth to apoptosis" to "in tumour cell lines diverted these cells from undergoing cell cycle arrest to cells entering the apoptosis pathway,...."
34	23	Change "H1 (the most variable)" to "H1 (the most variable by sequence)"
41	8	Change "(Oshima, Dinchuk et al. 1996)" to "(Oshima *et al.*, 1996)"
58	10	Change "tris base" to "Tris Base"
60	9	Change "Donkey anti-rabbit Ig" to "Donkey anti-rabbit IgG"
61	3	Insert the sentence "In some experiments, non-adherent cells were separated from adherent cells by decanting them."
66	4	Change "Taq" to "*Taq*"
69	4	Insert at the end of the paragraph the sentence "Synergism is defined as a response produced by a combination of two reagents, that is significantly greater than the sum of the individual response, as determined by the student *t*-test."
74	7	Change "3-fold after and 5-fold after 4 days" to " 2-fold after 2 days."
75	16	Change "consistent" to " accompanied by"
80	9	Insert after ".... not the case" "(Hague *et al.*, 1993; Heerdt *et al.*, 1998; Janson *et al.*, 1997; Nakano *et al.*, 1997)."
80	10	Insert "In addition, LIM1215 cells are from a HNPCC patient and therefore may differ from SW480 cells in other ways due to mismatch repair."
84	14	Insert after ".... middle panel)." the sentence "GAPDH is not modulated by butyrate (Nakano *et al.*, 1997)."
101	14	Insert after "....this hypothesis." the sentence "STS has been reported to synergize with butyrate in induction of apoptosis (Rickard et al., 1999), suggesting that its mechanism of action is distinct from that of butyrate."
102	11	Change "(data not shown)" to "(Archer *et al.*, 1998)."
115	2	Change "....has been accepted as" to "has been suggested to be"
118	8	Insert after ".... 200 μM." the sentence "These concentrations of NSAIDS were chosen based on published data (Piazza *et al.*, 1997)."
130	9	Insert after " LIM 1215 cells." the sentence "These concentrations of NDGA have been shown to block Lox activity (Kamitani *et al.*, 1998)."
134	1-2	Change " the cell suicide process of apoptosis (gene- directed cell death)" to "apoptosis"
144	19	At the beginning of the paragraph, insert the sentence "Previous studies from our laboratory (Zalewski *et al.*, 1993), have shown that treament of cells with 25 μM TPEN induces a moderate zinc depletion."
150	22	Insert the sentence "Background fluorescence was 21 pixels and has been already subtracted."
159	20	Insert the sentence "The effects of the 6 kDa fragment on CDK binding are not known, but since this fragment lacks the CDK binding motif this is unlikely."
162	23	Insert the sentence "It is also necessary to measure the histone acetylation after treatment with the various drugs, to exclude the role of histone deacetylation in butyrate resistance."

Figure 6.1&2		Change "PCRII" to *"pCRII"*
Figure 6.7		Insert at the end of the figure legend, the sentence "Due to the low transfection efficiencies, data were not corrected for % transfected cells."
Figure 7.8 & 7.9		Insert at the end of the figure legends, the phrase "significances are for comparisons between responses for the combination of reagents and the sum of responses by the individual reagents."
Figure 7.15		Change "Sw480" to "SW480"
Table 10.1		Change "Synergistic of" to "Synergistic or"
References		Remove endnote annotations from Huang et al and Nicholson et al. Extra references are listed below.

Additional References

Baker, S.J., Fearon, E.R., Nigro, J.M., Hamilton, S.R., Preisinger, A.C., Jessup, J.M., vanTuinen, P., Ledbetter, D.H., Barker, D.F., Nakamura, Y. and et al., Chromosome 17 deletions and p53 gene mutations in colorectal carcinomas. *Science*, **244,** 217-21 (1989).

Fuchs, C.S., Giovannucci, E.L., Colditz, G.A., Hunter, D.J., Stampfer, M.J., Rosner, B., Speizer, F.E. and Willett, W.C., Dietary fiber and the risk of colorectal cancer and adenoma in women. *N Engl J Med*, **340,** 169-76 (1999).

Jacobs, L.R., Relationship between dietary fiber and cancer: metabolic, physiologic, and cellular mechanisms. *Proc Soc Exp Biol Med*, **183,** 299-310 (1986).

Piazza, G.A., Rahm, A.K., Finn, T.S., Fryer, B.H., Li, H., Stoumen, A.L., Pamukcu, R. and Ahnen, D.J., Apoptosis primarily accounts for the growth-inhibitory properties of sulindac metabolites and involves a mechanism that is independent of cyclooxygenase inhibition, cell cycle arrest, and p53 induction. *Cancer Res*, **57,** 2452-9 (1997).

Rickard, K.L., Gibson, P.R., Young, G.P. and Phillips, W.A., Activation of protein kinase C augments butyrate-induced differentiation and turnover in human colonic epithelial cells in vitro. *Carcinogenesis*, **20,** 977-84 (1999).

Shanmugathasan, M. and Jothy, S., Apoptosis, anoikis and their relevance to the pathobiology of colon cancer. *Pathol Int*, **50,** 273-9 (2000).

Thompson, M.A., Rosenthal, M.A., Ellis, S.L., Friend, A.J., Zorbas, M.I., Whitehead, R.H. and Ramsay, R.G., c-Myb down-regulation is associated with human colon cell differentiation, apoptosis, and decreased Bcl-2 expression. *Cancer Res*, **58,** 5168-75 (1998).