

## Anti-cancer effects of butyrate: use of micro-array technology to investigate mechanisms

Elizabeth A. Williams\*, Jonathan M. Coxhead and John C. Mathers

Human Nutrition Research Centre, School of Clinical Medical Sciences, University of Newcastle upon Tyne, Newcastle upon Tyne NE1 7RU, UK

Epidemiological evidence suggests that a high intake of resistant starch and NSP protects against colo-rectal cancer. The mechanisms underlying this protection are thought to be mediated by the short-chain fatty acid butyrate, which is present in the colonic lumen in millimolar concentrations as a result of bacterial fermentation of carbohydrates that have resisted digestion in the small intestine. *In vitro* studies have shown that butyrate displays a host of chemo-preventative properties including increased apoptosis, reduced proliferation, down regulation of angiogenesis, enhanced immunosurveillance and anti-inflammatory effects in colo-rectal cancer cell lines. However, the molecular mechanisms underlying the apparent chemo-preventative actions of butyrate are largely unknown. The evidence supporting the role of butyrate as an anti-cancer agent is reviewed, with particular emphasis on those studies that have attempted to elucidate the mechanism of action of butyrate. Our understanding of the mechanistic action of butyrate and its role in cancer prevention is likely to advance considerably in this post-genomic era with the application of genomic and proteomic technologies. Studies are described that have used gene array and proteomic techniques to investigate the response of colo-rectal cancer cells to butyrate. These pioneering studies illustrate the potential of these technologies to help characterise the molecular responses of the cancer cell to butyrate, and to define the role of butyrate (and other nutrients) in the prevention of colo-rectal cancer.

### Butyrate: Micro-array techniques: Colo-rectal cancer

Butyrate is an abundant anion in colonic contents where it occurs at concentrations of 2–10 mM (Cummings *et al.* 1987). As with the other major short-chain fatty acids (acetate and propionate), butyrate is an end product of bacterial fermentation of carbohydrates that have escaped digestion in the small bowel. Higher intakes of these structurally-disparate fermentable carbohydrates, which include resistant starches, NSP and oligosaccharides, are associated with reduced risk of colo-rectal cancer (CRC) (Bingham, 1990; World Cancer Research Fund and the American Institute of Cancer Research, 1997). The mechanisms underlying this putative chemo-preventative effect are poorly understood, but the strongest evidence is based on the anti-neoplastic actions of butyrate. Most of this evidence comes from *in vitro* studies, but D'Argenio *et al.* (1996) showed that direct administration of butyrate into the colon of rats treated with the carcinogen azoxymethane reduced the number and size of tumours. The present review summarises the evidence for the tumour-suppressing

activity of butyrate (Fig. 1) and indicates the promise offered by transcriptomics (in the form of cDNA micro-array technology) and proteomics to help elucidate the mechanism of action of butyrate.

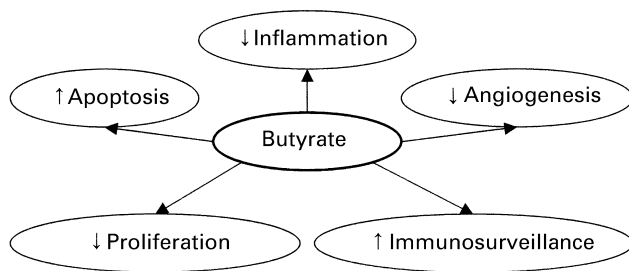
### Suppression of cell proliferation

For at least two decades it has been well established that butyrate in low millimolar concentrations suppresses the growth of a range of animal cells (Prasad & Sinha, 1976; D'Anna *et al.* 1980; Kruh, 1982; Wintersberger *et al.* 1983), including CRC cell lines (Whitehead *et al.* 1986; Gamet *et al.* 1992; Siavoshian *et al.* 1997). Exposure to butyrate results in arrest in the G<sub>1</sub> phase of the cell cycle and is associated with the induction of terminal differentiation (Leder & Leder, 1975; Siavoshian *et al.* 2000). Progression through the G<sub>1</sub> phase of the cell cycle requires inactivation of the retinoblastoma protein, a phosphoprotein of 105–114 kDa located in the nucleus (Weinberg, 1995). This inactivation

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**Abbreviations:** CRC, colo-rectal cancer; DAF, decay accelerating factor; IL, interleukin; TSA, Trichostatin A.

**\*Corresponding author:** Dr Liz Williams, fax +44 191 222 8684, email e.a.williams@ncl.ac.uk



**Fig. 1.** Potential mechanisms by which the anti-neoplastic actions of butyrate may be mediated.

occurs following phosphorylation of the retinoblastoma protein by cyclin D kinases 4 and 5 and the release of transcription factors, including those of the E2F family. Schwartz *et al.* (1998) reported that butyrate treatment (2 mM for up to 4 d) of LS174T colon cancer cells inhibited thymidine kinase activity (required for nucleotide synthesis in the S phase of the cell cycle) at the same time as inducing dephosphorylation of retinoblastoma protein and resulting in growth arrest. This growth arrest appears to be caused by induction of expression of the cyclin D kinase inhibitor p16 (Schwartz *et al.* 1998). Butyrate treatment of CRC cells stimulates expression of cyclin D3 (Siavoshian *et al.* 2000) and of the universal cyclin-dependent kinase inhibitor p21<sup>Waf1/Cip1</sup> (Chai *et al.* 2000) in a dose-dependent manner (Siavoshian *et al.* 1997). These increases in cyclin D3 and of p21 have been interpreted as markers of induction of differentiation (Steinman *et al.* 1994; Kiess *et al.* 1995). Kruh *et al.* (1995) reviewed the evidence that differentiation induced by butyrate alters the morphology and ultrastructure of tumour cells, including changes to cell surface mucopolysaccharides and glycolipids, resulting in the loss of most of the malignant characteristics of the cancerous cell.

### Induction of apoptosis

Resistance to death by apoptosis is one of the hallmarks of cancer cells (Hanahan & Weinberg, 2000) and this factor, together with maintained or enhanced rates of cell proliferation, contributes to expansion of the tumour mass. Hague *et al.* (1993) were the first to demonstrate that butyrate induced apoptosis in both adenoma and carcinoma cell lines, and reported that this induction of apoptosis was p53-independent (see also Mahyar-Roemer & Roemer, 2001). The latter is particularly interesting because p53 (often described as the 'guardian of the genome') is a transcription factor (Evan & Littlewood, 1998) that increases in concentration in response to DNA damage and results in growth arrest and repair, or in apoptosis of the damaged cell (Liu & Kulesz-Martin, 2001). At least 50 % of CRC tumours contain p53 mutations, rendering them susceptible to failure of apoptosis and increased accumulation of DNA damage. Heerdt *et al.* (1994) confirmed the ability of butyrate to induce apoptosis in colonic tumour cells and proposed that this process occurred via a terminal differentiation pathway. Increased expression of the differentiation markers E-cadherin and alkaline phosphatase was observed in CRC cell lines treated with butyrate (Butt *et al.* 1997). More recent studies have begun to dissect the molecular

mechanisms by which butyrate treatment results in tumour cell apoptosis, and have shown that butyrate up regulates expression of the pro-apoptotic protein BCL-2 homologous antagonist/killer (BAK) and induces caspase-3-mediated cleavage of target proteins, including poly-(ADP-ribose) polymerase (PARP) (Ruemmele *et al.* 1999; Clarke *et al.* 2001), the universal cyclin-dependent kinase inhibitor p21<sup>Waf1/Cip1</sup> (Chai *et al.* 2000) and adenomatous polyposis coli (APC). The latter is cleaved after Asp<sup>777</sup> to yield a characteristic and stable 90 kDa fragment (Browne *et al.* 1994, 1998; Webb *et al.* 1999).

### Anti-inflammatory actions

Intestinal diseases such as ulcerative colitis and Crohn's disease, which have an important inflammatory component in their pathology, carry an increased risk of CRC. Butyrate has anti-inflammatory effects (Inan *et al.* 2000; Saemann *et al.* 2000), which may be mediated by interference with the maturation of monocyte-derived dendritic cells (Saemann *et al.* 2002). Butyrate appears to shift the T helper-1–T helper-2 balance by inhibiting production of the pro-inflammatory cytokines interleukin (IL) 12, tumour necrosis factor- $\alpha$  and interferon- $\gamma$ , whilst having no effect on, or stimulating, the release of IL-6 and IL-10 (Saemann *et al.* 2000, 2002; Nancey *et al.* 2002). The molecular mechanism for this shift is unclear, although there is evidence of suppression of nuclear factor kappaB activation in some (Inan *et al.* 2000) but not all (Diakos *et al.* 2002) studies. Faust *et al.* (2001) have argued that the anti-inflammatory actions of butyrate in the intestinal mucosa are unlikely to occur by direct regulation of cytokine-induced anti-inflammatory protein expression. Recent data from Diakos *et al.* (2002) indicate that at relatively low concentrations (1 mM) butyrate inhibits production of the growth factor IL-2 (which is required for expansion of antigen-specific T cells) by decreasing nuclear binding of the transcription factor nuclear factor of activated T cells in both Jurkat cells and in primary T cells.

### Up regulation of immunosurveillance

Although still a controversial concept (for a review, see Armstrong & Mathers, 2000), it is becoming clearer that some chemo-preventative agents may act by up regulating immunosurveillance. The mechanisms for enhanced immunosurveillance could include better 'visualisation' of tumour cells as foreign by the immune system and/or attenuation of the ability of tumour cells to fight back against immune cells. Perrin *et al.* (1994) reported that the growth of PROb cancer cells, injected into the peritoneum of rats, was suppressed by concurrent treatment with butyrate and recombinant IL-2. The weak immunogenicity of PROb cells seemed to be enhanced by butyrate treatment making the tumour cells more susceptible to IL-2-activated natural killer cells. Butyrate may increase the effectiveness of other anti-cancer agents, as shown recently for actinomycin D in a mouse melanoma model (Giermasz *et al.* 2002). Butyrate treatment decreased expression of decay-accelerating factor (DAF) in three colon cancer cell lines (HT-29, Caco-2 and T-84; Andoh *et al.* 2002). DAF is expressed on the plasma membrane of CRC cells, forming a barrier to complement-

mediated clearance by the humoral immune response so that down regulation of DAF by butyrate appears to improve immunosurveillance (Andoh *et al.* 2002). Bonnotte *et al.* (1998) demonstrated that exposure of several human and rat CRC cell lines to butyrate *in vitro* increased their sensitivity to Fas ligand-mediated apoptosis in the absence of a change in expression of the Fas receptor on the surface of the target cells.

### Down regulation of angiogenesis

Expansion of solid tumours, including colon carcinomas, requires the production of a new blood supply (neovascularisation). As prevention of neovascularisation will limit the growth of tumours, angiogenesis has become a very attractive target for anti-tumour agents. The cyclooxygenase-2 selective inhibitor celecoxib, which has been shown to suppress the appearance of colonic polyps in individuals with familial adenomatous polyposis (Steinbach *et al.* 2000), may act in part via inhibition of angiogenesis. There is now evidence that butyrate may also have anti-angiogenic effects. Chemokines and their receptors are involved in angiogenesis, and Jordan *et al.* (1999) showed that HT-29 cells expressed the chemokine receptor CXCR4. However, within 3 h of treatment with 5 mM-butyrates CXCR4 expression by HT-29 cells was inhibited completely (Jordan *et al.* 1999). Treatment of the same cell line with 2 mM-butyrates down regulated expression of two angiogenesis-related proteins i.e. vascular endothelial growth factor, the most potent angiogenic factor, and hypoxia-inducible factor-1 $\alpha$  (Pellizzaro *et al.* 2002).

### Functional genomics and proteomics

Over recent years the sequencing of the whole genome of different organisms, including man, has provided the foundation for several powerful new biotechnologies offering insight into: (a) the molecular characteristics of the cell; (b) the regulation of cellular activity; (c) the response of the cell to its environment; (d) cellular defects in disease states. Genomics, transcriptomics and proteomics are all based on the faithful transcription of DNA from the coding region of the gene into a complementary single-stranded mRNA molecule and subsequent translation into the protein (Fig. 2). Application of these technologies is revolutionising the biosciences, but it is in medical sciences where they are likely to have their biggest impact (Mohr *et al.* 2002). Functional genomics is being used to characterise the molecular events occurring in disease processes (Kitahara *et al.* 2001; Luo *et al.* 2001), to identify polymorphisms in genes that may be associated with increased (or decreased) risk of specific diseases (Hacia & Collins, 1999; Sapolsky *et al.* 1999) and to search for biomarkers of disease (Van Eyk, 2001; Jones *et al.* 2002). In addition, these technologies offer exciting opportunities to explore and characterise the mechanistic action of nutrients at a molecular level. The following is a brief overview of the technologies themselves and an indication of how their application has begun to be used to help elucidate the mechanism(s) underlying the anti-neoplastic action of butyrate.

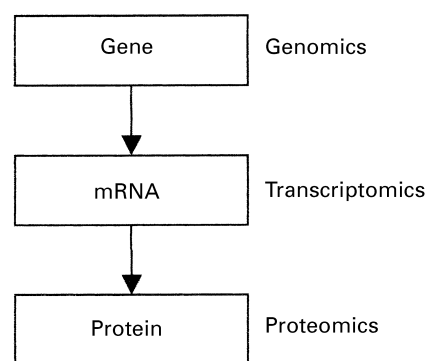


Fig. 2. Relationships between the 'omics' technologies.

### Genomics and transcriptomics

DNA array technology allows the expression of thousands of genes to be analysed simultaneously. This high-throughput approach is facilitating rapid advances in genomics and transcriptomics. The principle underlying the array technologies is hybridisation between complementary single-stranded nucleic acid sequences exploiting the specific relationship between A and T, and G and C (Southern *et al.* 1999). The latest micro-arrays consist of densely-packed spots of single-strand cDNA or oligonucleotides complementary to the sequence of interest immobilized onto a substrate, e.g. glass or nitrocellulose. mRNA is extracted from cells, reverse-transcribed using fluorescently-labelled nucleotides and hybridised to the array. The array is laser-scanned and expression levels determined from the intensity of the fluorescence at each spot. The results are processed through a database and data mining tools used for analysis (Duggan *et al.* 1999). Such experiments generate massive amounts of expression data, the interpretation of which is challenging and dependent largely on the quality of genomic databases and of the software tools available. Good experimental design and careful attention to experimental protocols are critical to the success of array experiments. For example, homogeneous populations of cells may be difficult or impossible to extract from heterogeneous biopsy material, so that interpretation of the gene expression data may become equivocal. In addition, without appropriate precautions, less-abundant mRNA may fall below the limits of detection. Array experiments are often complemented by more conventional molecular approaches, e.g. Northern or Western blotting and reverse transcription-polymerase chain reaction to confirm the expression data and/or to investigate the kinetics of expression. mRNA expression does not necessarily correlate with protein expression (Anderson & Seilhamer, 1997), and does not account for post-translational modification of proteins, so that an integrated approach using transcriptomics and proteomics can provide a powerful insight into cellular activity.

### Proteomics

The term proteome was coined by Wasinger *et al.* (1995) to describe the protein complement of the genome. Proteomics

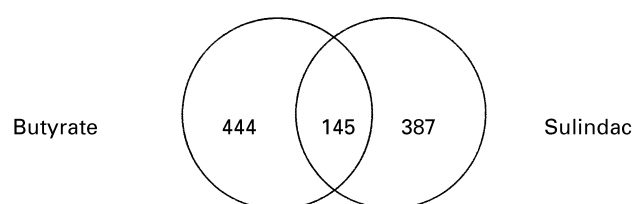
is the study of the protein properties of the cell, encompassing protein expression, protein interactions and post-translational modifications (Blackstock & Weir, 1999). At the core of the technology is two-dimensional gel electrophoresis, which separates proteins according to molecular weight in one dimension and isoelectric charge in the other (O'Farrell, 1975). The use of large two-dimensional gels allows thousands of protein spots to be resolved in a single gel (Klose, 1999). Gels may be stained using Coomassie blue, silver staining or a fluorescent dye, allowing visualisation of proteins, image analysis and spot detection. The position of a protein spot on the gel may give clues to its identification, but unambiguous protein identification depends largely on MS. For identification a protein spot is selected from the two-dimensional gel, digested with an appropriate protease and its composite peptides identified according to their mass using MS. The pattern of peptide masses provides a unique protein fingerprint that can be used to search protein databases to identify the protein itself. Proteins not identified through this approach are subjected to more advanced MS methods, e.g. matrix-assisted laser desorption-ionisation time-of-flight (MALDI-TOF) allowing partial peptide sequencing. Peptides can then be characterised using nucleic acid sequence databases to search for their sequence tags. Major improvements in MS techniques have led to improved protein identification and detection of post-translational modification which, coupled with the development of protein and nucleic acid databases, have resulted in considerable advances in the field.

### Exploring the anti-neoplastic properties of butyrate using functional genomics

The application of functional genomics to define the mechanisms underlying the neoplastic properties of butyrate has thus far been confined to homogeneous cell cultures. These pioneering studies will be reviewed.

#### *The first in the field*

The first published study that used micro-array technology to investigate the effect of butyrate on the transcriptome of colonocytes was that of Mariadason *et al.* (2000). The transcriptional response of SW620 colon carcinoma cell line to 5 mM-butyrate was compared with responses to three other compounds, i.e. Trichostatin A (TSA; an effective histone deacetylase inhibitor), sulindac (a non-steroidal anti-inflammatory drug) and curcumin (a dietary chemoprotective agent). Expression profiles of 8063 gene sequences were analysed using micro-array technology at six time points up to 48 h post-treatment, and only those genes whose expression, once modified, was sustained until the end of exposure to the agent were included in the analysis. The results demonstrate a complex cascade of reprogramming of SW620 colonic epithelial cells in response to butyrate over time. Genes were recruited rapidly in response to butyrate exposure, with some changes in gene expression observed within 30 min. Subsequent time points were characterised by a progressive expansion of the population of differentially-expressed genes that began to



**Fig. 3.** Summary of the number of genes differentially expressed in SW620 cells following 48 h exposure to either 5 mM-butyrate or 1.6 mM-sulindac. (From Mariadason *et al.* 2000.)

moderate after 16 h. Over the 48 h a staggering 589 genes were reprogrammed (256 up regulated, 333 down regulated) in the presence of butyrate. Almost as many (534) genes were differentially expressed on exposure to sulindac, with less extensive changes following treatment with TSA and curcumin.

Detailed interrogation of the data showed that, despite the similarities in the phenotypic response of SW620 cells to the butyrate and to the non-steroidal anti-inflammatory drug sulindac, the underlying genetic responses were profoundly different. Both butyrate and sulindac induce G<sub>0</sub>-G<sub>1</sub> cell cycle arrest, trigger an apoptotic cascade and up regulate  $\beta$ -catenin-T cell factor. Of the more than 500 genes that were differentially expressed in response to each of the agents, 145 genes were mutually responsive to both butyrate and sulindac exposure (Fig. 3). However, only ninety-two of these genes responded in the same manner to both compounds, with thirty-six up regulated and fifty-six down regulated. The compounds had opposite actions on the remaining fifty-three genes. The genes responsive to butyrate exposure were most comparable with the gene profile evoked by TSA treatment. Butyrate, sulindac and TSA all induce G<sub>0</sub>-G<sub>1</sub> cell cycle arrest, yet there was more similarity between the cell cycle genes altered in response to butyrate and TSA than in response to sulindac. The greatest differences in response compared with that to butyrate were seen for curcumin, which arrests cells in G<sub>2</sub>-M. The authors hypothesised that the activity of butyrate and TSA as histone deacetylase inhibitors could account for the similarity in gene profiles. Both butyrate and TSA inhibited histone deacetylase (observed as an altered acetylated histone H4:histone H1 value, determined by Western blotting), but the kinetics profile of histone hyperacetylation differed for the two agents. These differential kinetics allowed the identification of two clusters of genes (not differentially altered by either sulindac or curcumin) that were activated in tandem with histone acetylation, suggesting that the changes in these genes occur as a consequence of inhibition of histone deacetylase activity. Mariadason *et al.* (2000) conclude that, in the context of CRC chemo-prevention, agents such as butyrate that induce death of tumour cells by reprogramming them along a 'natural' maturation pathway may offer greater advantages in terms of safety than, for example sulindac, which also induces tumour cell apoptosis but which is associated with side-effects such as mucosal ulceration when used over long periods. In addition, the revelation by micro-array analysis that two agents may induce tumour cell apoptosis by different pathways suggests the possibility of synergy, and opens opportunities for use of combinations of chemo-preventative agents.



### Choosing the most informative time point

Before deciding on the conditions for an array experiment, Iacomino *et al.* (2001) confirmed that 4 mM-butyrate suppressed cell growth and induced differentiation in the colonic adenocarcinoma cell line HT29. Butyrate-treated cells accumulated in the G<sub>1</sub> phase of the cell cycle and there was increased expression of the differentiation marker alkaline phosphatase. Protein expression of the cyclin-dependent kinase inhibitor p21<sup>Waf1/Cip1</sup> was elevated, whilst the oncogene c-myc was reduced at 48–72 h. Based on these findings Iacomino *et al.* (2001) chose to perform their array experiment at 72 h incubation with butyrate when cell cycle arrest and cell differentiation were maximal. They used an Atlas cDNA array to characterise and quantify the expression of 588 genes, including those involved in cell cycle regulation, signal transduction, apoptosis, DNA synthesis and repair, transcription and the stress response. Of these 588 genes, the HT-29 cells expressed 119, with sixty genes being differentially expressed (thirty-nine up regulated and twenty-one down regulated) in response to butyrate. In this study a twofold change in expression was considered significant. A cluster of genes regulating apoptosis, DNA synthesis, repair and recombination were among those up regulated, whilst a group of oncogenes, cell cycle regulators and transcription factors were down regulated. Reverse transcription–polymerase chain reaction was performed on a random selection of genes to confirm the array results, and on a sub-set of genes that were strongly responsive to butyrate.

Among the interesting findings of this study was the observation that butyrate treatment up regulated expression of a number of genes involved in the detoxification of xenobiotics. This finding suggests that increased butyrate supply to colonocytes might modulate the damage caused by carcinogens and/or alter the sensitivity to anti-tumour drugs. The response of some genes was unexpected, leading the authors to postulate on the role of these genes in the butyrate response. For example, they describe pro- and anti-apoptotic mRNA being concurrently up regulated, a feature that could be explained by deranged activity of cancer cells. In single-time-point micro-array studies such as this study, primary responses to the intervention cannot be distinguished from downstream events, and early events may be missed entirely if they occur transiently in advance of the chosen sampling time point. These possibilities complicate the interpretation of such data.

### Focusing on mRNA expression to avoid confounding by downstream events

Della Ragione *et al.* (2001) combined aspects of the previous two studies to provide an elegant solution to the problem of downstream events masking the direct effect of butyrate on gene expression. These authors investigated the effects of treatment with butyrate or TSA in the presence of cycloheximide, to inhibit *de novo* protein synthesis, on the expression profile of HT29 cells. In this way they were able to observe transcriptional effects only. HT29 cells were treated with 2 mM-butyrate or 0.3 μM-TSA in the presence of cycloheximide for 5 h. Using the Atlas cDNA array of

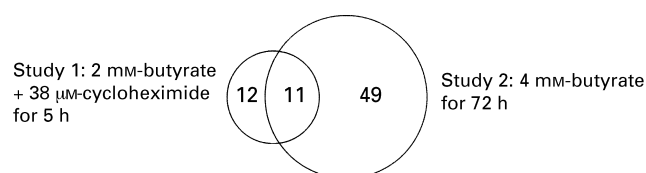
588 genes, it was observed that the expression of twenty-three genes was modulated in an identical manner (two down regulated and the remainder up regulated) by both butyrate and TSA. Since TSA is a well-characterised histone deacetylase inhibitor, these data provide strong evidence that the initial responses (up to 5 h after exposure) to butyrate treatment occur via histone deacetylation inhibition.

Increased acetylation of histones would be expected to open up the chromatin structure and to increase transcription (Ballestar & Esteller, 2002), so the up regulation of expression of twenty-one genes accords with this hypothesis (Della Ragione *et al.* 2001). What is less clear is the extent to which there is selectivity in this response. In other words, does butyrate treatment lead to a generalised hyperacetylation of DNA? The available evidence obtained using conventional gene expression methods (Kruh *et al.* 1995) and the more recent micro-array techniques (Mariadason *et al.* 2000; Della Ragione *et al.* 2001; Iacomino *et al.* 2001) suggest that exposure to butyrate up regulates expression of only a relatively small number of genes, but it should be remembered that the cDNA micro-array techniques are insensitive to small changes in expression (usually less than a twofold change).

In the study carried out by Della Ragione *et al.* (2001) only two of the differentially-expressed genes had been reported to be modulated by butyrate in previous studies. The remainder of the genes, whose functions included cell cycle regulation and DNA repair, may be potentially fruitful areas for further investigations. These authors used Northern blotting and reverse transcription–polymerase chain reaction to confirm mRNA data, and Western blotting to examine protein expression of two genes i.e. *Tob-1* (an anti-proliferation mediator that acts synergistically with p21) and *GATA-2* (a nuclear transcription factor). In addition, they provided direct evidence of the anti-proliferative properties of *Tob-1* by transfecting HT29 cells with *Tob-1* cDNA and observing a reduction in [<sup>3</sup>H]thymidine incorporation that correlated with the extent of *Tob-1* protein expression.

### Concordance between array experiments

Iacomino *et al.* (2001) and Della Ragione *et al.* (2001) both investigated the response of HT29 cells to butyrate exposure using the same ATLAS cDNA array. Of the twenty-three genes induced after 5 h exposure to 2 mM-butyrate in the presence of cycloheximide in the experiment of Della Ragione *et al.* (2001), eleven were also induced after 72 h exposure to 4 mM-butyrate in the study of Iacomino *et al.* (2001) (Fig. 4). Of the eleven genes differentially expressed



**Fig. 4.** The number of genes differentially expressed by HT29 cells exposed to butyrate. Study 1, Della Ragione *et al.* (2001); study 2, Iacomino *et al.* (2001).

in both experiments nine were up regulated in both studies and two were up regulated after 5 h (Della Ragione *et al.* 2001) but down regulated after 72 h (Iacomino *et al.* 2001). Whilst aspects of the experimental conditions differed (butyrate concentration, time-frame and use of cycloheximide), the similarities in the gene profiles in response to butyrate (Table 1) offer potential targets for further study.

Several scenarios could explain the differences in gene expression in the two experiments including: (a) genes activated in the absence of cycloheximide may be downstream events; (b) there may be an overall expansion of the number of differentially-expressed genes between 5 and 72 h (Mariadason *et al.* 2000); (c) genes may be activated by butyrate in a concentration-dependent manner.

**Table 1.** Genes in HT29 cells whose expression was modified by butyrate treatment

Description	GenBank accession no.	Study*	Direction of change
<b>Oncogenes, tumour suppressor and cell cycle control proteins</b>			
Cyclin-dependent kinase inhibitor 1A (p21 <sup>Cip1</sup> )	U09579	1	↑
Growth arrest and DNA damage inducible protein (GADD45)	M60974	1	↑
Lactoferrin delta	U84119	1	↓
Mapkap kinase	U09578	1	↓
Tyrosine protein kinase SKY (TYRO3)	D17517	2	↑
Ezrin	X51521	2	↑
Prothymosin alpha (Pro-T-α)	M26708	2	↑
SHB adaptor protein (Src homology 2 protein)	X75342	2	↑
CDK4 inhibitor (p16-INK4)	L27211	2	↑
PCNA P120	X55504	2	↓
V-ERBA-related protein EAR-2	X12794	2	↓
Urea transporter RACH1 (HUT11)	U35735	2	↓
C-JUN proto-oncogene (JUN)	J04111	2	↓
40S ribosomal protein S19	M81757	2	↓
Proto-oncogene RHOA, multi-drug resistance protein	L25080	1 and 2	↑
TOB-1	D38305	1 and 2	↓
CD2-related protein kinase PISSLRE	L33264	1 and 2	↑↓
Cell division control protein (P55CDC)	U05340	1 and 2	↑↓
<b>Ion channel, stress-response proteins, transport-protein modulators, effectors and intracellular transducers</b>			
Protein kinase C delta type (PKCδ)	D10495	1	↑
Epithelial discoidin domain receptor 1 (TRKE)	X74979	1	↑
Proliferation-associated gene (PAG)	X67951	1	↑
TR3 orphan receptor (NAK1)	L13740	1	↑
Guanine nucleotide regulatory protein (NET1)	U02081	1	↑
EBI	U24166	1	↑
Urokinase type plasminogen activator surface receptor (u-PAR)	U08839	2	↑
Extracellular signal-regulated kinase 3 (ERK3)	M80692	2	↑
p38 Mitogen-activated protein kinase (p53 MAP kinase)	L35253	2	↑
Natural killer cell-enhancing factor B (NKEFB)	L19185	2	↑
Heat-shock protein 40 (HSP40)	D49547	2	↑
cAMP-dependent protein kinase type 1-β regulatory chain	M65066	2	↑
Transducin β-2 subunit	M36429	2	↓
Tyrosine-protein kinase receptor EPH	M18391	2	↓
C-X-C chemokine receptor type 4 (HM89)	D10924	1 and 2	↑
Heat-shock 27kDa protein (HSP27)	X54079	1 and 2	↑
Heat-shock 70 kDa protein (HSP70)	M11717	1 and 2	↑
Tyrosine-protein kinase receptor EPH-3 (EPHB3)	X75208	1 and 2	↑↓
<b>Apoptosis-related proteins, DNA synthesis, repair and recombination proteins</b>			
Glutathione S transferase θ 1 (GST-T-1)	X79389	1	↑
Glutathione S transferase Pi (GST-pi-1)	X15480	2	↑
Glutathione S transferase A1 (GST-A-1)	M25627	2	↑
WSL-LR;WSL-S1;WSL-S2 protein	Y09392	2	↑
MUTL ( <i>E. coli</i> ) homologue 1 (hMLH1)	U07418	2	↑
Defender against cell death 1 (DAD-1)	D15057	2	↑
Replication factor C. 37 kDa subunit	M87339	2	↑
Growth factor receptor-bound protein 2 (GRB2)	L29511	2	↑
Cell death protein RIP	U25994	2	↑
Fast kinase (Fas-activated serine–threonine kinase)	X86779	2	↑
Glutathione reductase	X15722	2	↑
Cytochrome P450 reductase	S90469	1 and 2	↑

**Table 1.**

Description	GenBank accession no.	Study*	Direction of change
<b>DNA binding and transcription factors</b>			
Transcriptional regulator ISGF3 $\gamma$ subunit	M87503	2	↑
Zinc finger X chromosomal protein	X59738	2	↑
Kruppel-related zinc finger protein HTF10	L11672	2	↑
Homeo box C1 protein	M16937	2	↑
Sterol regulatory element-binding protein (CNPB)	M28372	2	↑
Transcription factor ZFM1, alternatively spliced	D26121	2	↑
CUT (Drosophila)-like 1 (CCAAT-displacement protein)	L12579	2	↓
Zinc finger protein homologous to ZFP-36 in mouse	M92843	2	↓
GATA-binding protein 2 (GATA-2)	M68891	1 and 2	↑
<b>Cell receptors, interleukin and interferon receptors, hormone receptors, neurotransmitter receptors, cell surface antigens and adhesion</b>			
Protein-tyrosine kinase receptor ERBB-3	M29366	2	↓
Interleukin-5 receptor $\alpha$ chain	M75914	2	↓
Corticotropin-releasing factor receptor	X72304	2	↓
Neuromedin-B receptor (NMB-R)	M73482	2	↓
Intercellular adhesion molecule-1 (ICAM-1)	J03132	1 and 2	↑
<b>Extracellular cell signalling and communication proteins, interleukins and interferons, hormones</b>			
Placental ribonuclease inhibitor (RAI)	M36717	1	↑
Hepatoma-derived growth factor	D16431	2	↑
Placental growth factor (P1GF)	X54936	2	↑
Glucose-6-phosphate isomerase (neuroleukin)	K03515	2	↑
Interleukin 10 (IL-10)	M57627	2	↑
Acyl-CoA-binding protein	M14200	2	↑
Interleukin 1 $\beta$ (IL-1)	K02770	2	↑
Insulin-like growth factor-binding protein 1	M31145	2	↓
Vascular endothelial growth factor (VEGF)	M32977	2	↓
Interleukin 13	L06801	2	↓

↑, Genes up regulated; ↓, genes down regulated. \*Study 1, genes differentially expressed in HT29 cells treated for 5 h with 2 mM-butyrate in the presence of 36  $\mu$ M-cycloheximide (Della Ragione *et al.* 2001); study 2, genes differentially expressed in HT29 cells treated for 72 h with 4 mM-butyrate (Iacomino *et al.* 2001).

### Applying the proteomics approach

Tan *et al.* (2002) used proteomics to help elucidate the mechanism of action of butyrate in colonocytes. Total cell lysates from HT29 cells treated with or without butyrate (5 mM for 24 h) were analysed by two-dimensional gel electrophoresis. The gel resolved over 1000 proteins, and comparison of the gels derived from butyrate-treated and control cells revealed regions of the gel showing differential protein expression patterns. MALDI-TOF MS was then used to identify thirty-five of the differentially-expressed spots. The proteins were found to be predominantly of cytosolic or mitochondrial origin. The authors reviewed the putative functions of many of the proteins differentially expressed in relation to butyrate and observed that, in addition to altered expression of proteins involved in the cell cycle, apoptosis and transcriptional regulation (the usual suspects), there was also altered expression of components of the ubiquitin-proteasome system. Tan *et al.* (2002) hypothesised that butyrate may play a role in regulation of these fundamental cell processes by altered proteolysis of ubiquitinated cellular proteins. For example, it is known that expression of p21<sup>Waf1/Cip1</sup> (involved in butyrate-mediated G<sub>0</sub>-G<sub>1</sub> arrest) shows proteasome-dependent regulation (Blagosklonny *et al.* 1996).

### Conclusions and future developments

These studies demonstrate how transcriptomic and proteomic experiments can help to elucidate the mechanisms underlying the chemo-preventative properties of butyrate. This work is in its infancy and, given the considerable differences in experimental protocol (e.g. cell line, dose of butyrate, time of exposure, level of expression (mRNA v. protein)) used by the different authors, it is unsurprising that there are marked differences in the genes identified as being differentially expressed. However, there is consistent evidence of suppression of CRC cell growth and enhancement of apoptosis, with growing support for the hypothesis that inhibition of histone deacetylation is the probable first step in the molecular action of butyrate.

Expression profiling at the mRNA and protein levels is now established and it offers a powerful route to increased understanding of mechanisms underlying the anti-neoplastic effects of butyrate and other food-derived agents. Rather than focusing on one or a small handful of specific genes or proteins (often chosen largely for pragmatic reasons), transcriptomic and proteomic approaches allow us to consider all the genes transcribed in a cell at a given instant. The challenges are to design protocols that maximise the interpretable information from such studies and to use these data to develop and test specific mechanistic hypotheses.

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### References

- Anderson L & Seilhamer J (1997) A comparison of selected mRNA and protein abundances in human liver. *Electrophoresis* **18**, 533–537.
- Andoh A, Shimada M, Araki Y, Fujiyama Y & Bamba T (2002) Sodium butyrate enhances complement-mediated cell injury via down-regulation of decay-accelerating factor expression in colonic cancer cells. *Cancer Immunology and Immunotherapy* **50**, 663–672.
- Armstrong F & Mathers JC (2000) Kill and cure: dietary augmentation of immune defences against colon cancer. *Proceedings of the Nutrition Society* **59**, 215–220.
- Ballestar E & Esteller M (2002) The impact of chromatin in human cancer: linking DNA methylation to gene silencing. *Carcinogenesis* **23**, 1103–1109.
- Bingham SA (1990) Mechanisms and experimental and epidemiological evidence relating dietary fibre (non-starch polysaccharides) and starch to protection against large bowel cancer. *Proceedings of the Nutrition Society* **49**, 153–171.
- Blackstock WP & Weir MP (1999) Proteomics: quantitative and physical mapping of cellular proteins. *Trends in Biotechnology* **17**, 121–127.
- Blagosklonny MV, Wu GS, Omura S & el-Deiry WS (1996) Proteasome-dependent regulation of p21<sup>Waf1/Cip1</sup> expression. *Biochemical and Biophysical Research Communications* **227**, 564–569.
- Bonnotte B, Favre N, Reveneau S, Micheau O, Droin N, Garrido C, Fontana A, Chauffert B, Solary E & Martin F (1998) Cancer cell sensitization to Fas-mediated apoptosis by sodium butyrate. *Cell Death and Differentiation* **5**, 480–487.
- Browne SJ, Macfarlane M, Cohen GM & Paraskeva C (1998) The adenomatous polyposis coli protein and retinoblastoma proteins are cleaved early in apoptosis and are potential substrates for caspases. *Cell Death and Differentiation* **5**, 206–213.
- Browne SJ, Williams AC, Hague A, Butt AJ & Paraskeva C (1994) Loss of APC protein expressed by human colonic epithelial cells and the appearance of a specific low-molecular-weight form is associated with apoptosis *in vitro*. *International Journal of Cancer* **59**, 56–64.
- Butt AJ, Hague A & Paraskeva C (1997) Butyrate- but not TGF $\beta$ <sub>1</sub>-induced apoptosis of colorectal adenoma cells is associated with increased expression of the differentiation markers E-cadherin and alkaline phosphatase. *Cell Death and Differentiation* **4**, 725–734.
- Chai F, Evdokiou A, Young GP & Zalewski PD (2000) Involvement of p21<sup>Waf1/Cip1</sup> and its cleavage by DEVD-caspase during apoptosis of colorectal cancer cells induced by butyrate. *Carcinogenesis* **21**, 7–14.
- Clarke KO, Feinman R & Harrison LE (2001) Tributyrin, an oral butyrate analogue, induces apoptosis through activation of caspase-3. *Cancer Letters* **171**, 57–65.
- Cummings JH, Pomare EW, Branch WJ, Naylor CP & Macfarlane GT (1987) Short chain fatty acids in human large intestine, portal, hepatic and venous blood. *Gut* **28**, 1221–1227.
- D'Anna JA, Tobey RA & Gurley LR (1980) 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**, 2656–2671.
- D'Argenio G, Cosenza V, Delle Cave M, Iovino P, Della Valle N, Lombardi G & Mazzacca G (1996) Butyrate enemas in experimental colitis and protection against large bowel cancer in a rat model. *Gastroenterology* **110**, 1727–1734.
- Della Ragione F, Criniti V, Della Pietra V, Borriello A, Oliva A, Indaco S, Yamamoto T & Zappia V (2001) Genes modulated by histone acetylation as new effectors of butyrate activity. *FEBS Letters* **499**, 199–204.
- Diakos C, Prieschl EE, Saemann M, Novotny V, Bohmig G, Csonga R, Baumruker T & Zlabinger GJ (2002) Novel mode of interference with nuclear factor of activated T-cells regulation in T-cells by the bacterial metabolite *n*-butyrate. *Journal of Biological Chemistry* **277**, 24243–24251.
- Duggan DJ, Bittner M, Chen Y, Meltzer P & Trent JM (1999) Expression profiling using cDNA microarrays. *Nature Genetics* **21**, 10–14.
- Evan G & Littlewood T (1998) A matter of life and cell death. *Science* **281**, 1317–1322.
- Faust D, Hormann S, Friedrich-Sander M, Milovic V & Stein J (2001) Butyrate and the cytokine-induced alpha1-proteinase inhibitor release in intestinal epithelial cells. *European Journal of Clinical Investigation* **31**, 1060–1063.
- Gamet L, Daviaud D, Denis-Pouxviel C, Remesy C & Murat JC (1992) Effects of short-chain fatty acids on growth and differentiation on the human colon-cancer cell line HT-29. *International Journal of Cancer* **52**, 286–289.
- Giermasz A, Makowski M, Nowis D, Jalili A, Maj M, Dabrowska A, Czajka A, Jakobisiak M & Golab J (2002) Potentiated anti-tumour effects of butyrate and actinomycin D in melanoma model in mice. *Oncology Reports* **9**, 199–203.
- Hacia JG & Collins FS (1999) Mutational analysis using oligonucleotide microarrays. *Journal of Medical Genetics* **36**, 730–736.
- Hague A, Manning AM, Hanlon KA, Huschtscha LI, Hart D & Paraskeva C (1993) 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. *International Journal of Cancer* **55**, 498–505.
- Hanahan D & Weinberg RA (2000) The hallmarks of cancer. *Cell* **100**, 57–70.
- Heerdt BG, Houston MA & Augenlicht LH (1994) Potentiation by specific short-chain fatty acids of differentiation and apoptosis in human colonic carcinoma cell lines. *Cancer Research* **54**, 3288–3294.
- Iacomino G, Tecce M, Grimaldi C, Tosto M & Russo GL (2001) Transcriptional response of a human colon adenocarcinoma cell line to sodium butyrate. *Biochemical and Biophysical Research Communications* **285**, 1280–1289.
- Inan MS, Rasoulpour RJ, Yin L, Hubbard AK, Rosenberg DW & Giardina C (2000) The luminal short-chain fatty acid butyrate modulates NF-kappaB activity in a human colonic epithelial cell line. *Gastroenterology* **118**, 724–734.
- Jones MB, Krutzsch H, Shu H, Zhao Y, Liotta LA, Kohn EC & Petricoin EF (2002) Proteomic analysis and identification of new biomarkers and therapeutic targets for invasive ovarian cancer. *Proteomics* **2**, 76–84.
- Jordan NJ, Kolios G, Abbot SE, Sinai MA, Thompson DA, Petraki K & Westwick J (1999) Expression of functional CXCR4 chemokine receptors on human colonic epithelial cells. *Journal of Clinical Investigation* **104**, 1061–1069.
- Kiess M, Gill RM & Hamel PA (1995) Expression of the positive regulator of cell cycle progression, cyclin D3, is induced during differentiation of myoblasts into quiescent myotubules. *Oncogene* **10**, 159–166.
- Kitahara O, Furukawa Y, Tanaka T, Kilhara C, Ono K, Yanagawa R, Nita ME, Takagi T, Nakamura Y & Tsunoda T (2001) Alterations of gene expression during colorectal carcinogenesis



- revealed by cDNA microarrays after laser-capture microdissection of tumor tissues and normal epithelia. *Cancer Research* **61**, 3544–3549.
- Klose J (1999) Large-gel 2-D electrophoresis. *Methods in Molecular Biology* **112**, 147–172.
- Kruh J (1982) Effects of sodium butyrate, a new pharmacological agent, on cells in culture. *Molecular and Cellular Biochemistry* **42**, 65–82.
- Kruh J, Defer N & Tichonicky L (1995) Effects of butyrate on cell proliferation and gene expression. In *Physiological and Clinical Aspects of Short-chain Fatty Acids*, pp. 275–288 [JH Cummings, JL Rombeau and T Sakata, editors]. Cambridge: Cambridge University Press.
- Leder A & Leder P (1975) Butyric acid, a potent inducer of erythroid differentiation in cultured erythroleukemic cells. *Cell* **5**, 319–322.
- Liu Y & Kulesz-Martin M (2001) p53 protein at the hub of cellular DNA damage response pathways through sequence-specific and non-sequence-specific DNA binding. *Carcinogenesis* **22**, 851–860.
- Luo J, Duggan DJ, Chen Y, Sauvageot J, Ewing CM, Bittner ML, Trent JM & Isaacs WB (2001) Human prostate cancer and benign prostatic hyperplasia: molecular dissection by gene expression profiling. *Cancer Research* **61**, 4683–4688.
- Mahyar-Roemer M & Roemer K (2001) p21 Waf1/Cip1 can protect human colon carcinoma cells against p53-dependent and p53-independent apoptosis induced by natural chemopreventive and therapeutic agents. *Oncogene* **20**, 3387–3398.
- Mariadason J, Corner GA & Augenlicht LH (2000) Genetic reprogramming in pathways of colonic cell maturation induced by short chain fatty acids: Comparison with trichostatin A, sulindac, and curcumin and implications for chemoprevention of colorectal cancer. *Cancer Research* **60**, 4561–4572.
- Mohr S, Leikauf GD, Keith G & Rihn BH (2002) Microarrays as cancer keys: an array of possibilities. *Journal of Clinical Oncology* **20**, 3165–3175.
- Nancey S, Bienvenu J, Coffin B, Andre F, Decos L & Flourie B (2002) Butyrate strongly inhibits in vitro stimulated release of cytokines in blood. *Digestive Diseases and Sciences* **47**, 921–928.
- O'Farrell PH (1975) High resolution two-dimensional electrophoresis of proteins. *Journal of Biological Chemistry* **250**, 4007–4021.
- Pellizzaro C, Coradini D & Daidone GG (2002) Modulation of angiogenesis-related proteins synthesis by sodium butyrate in colon cancer cell line HT29. *Carcinogenesis* **23**, 735–740.
- Perrin P, Cassagnau E, Burg C, Party Y, Vavasseur F, Harb J, Le Pendu J, Douillard J-Y, Galmiche J-P, Bornet F & Meflah K (1994) An interleukin 2/sodium butyrate combination as immunotherapy for rat colon cancer peritoneal carcinomatosis. *Gastroenterology* **107**, 1397–1408.
- Prasad KN & Sinha PK (1976) Effect of sodium butyrate on mammalian cells in culture. *In Vitro* **12**, 125–132.
- Ruemmele FM, Dionne S, Qureshi I, Sarma DSR, Levy E & Seidman EG (1999) Butyrate mediates Caco-2 cell apoptosis via up-regulation of pro-apoptotic BAK and inducing caspase-3 mediated cleavage of poly-(ADP-ribose) polymerase (PARP). *Cell Death and Differentiation* **6**, 729–735.
- Saemann MD, Bohmig GA, Osterreicher CH, Burtscher H, Parolini O, Diakos C, Stockl J, Horl WH & Zlabinger GJ (2000) Anti-inflammatory effects of sodium butyrate on human monocytes: potent inhibition of IL-12 and up-regulation of IL-10 production. *FASEB Journal* **14**, 2380–2382.
- Saemann MD, Parolini O, Bohmig GA, Kelemen P, Krieger PM, Neumuller J, Kanrr K, Kammlaander W, Horl WH, Diakos C, Stuhlmeier K & Zlabinger GJ (2002) Bacterial metabolite interference with maturation of human monocyte-derived dendritic cells. *Journal of Leukocyte Biology* **71**, 238–246.
- Sapolsky RJ, Hsie L, Berno A, Ghandour G, Mittmann M & Fan JB (1999) High-throughput polymorphism screening and genotyping with high-density oligonucleotide arrays. *Genetic Analysis* **14**, 187–192.
- Schwartz B, Avivi-Green C & Polak-Charcon S (1998) Sodium butyrate induces retinoblastoma protein dephosphorylation, p16 expression and growth arrest of colon cancer cell. *Molecular and Cellular Biochemistry* **188**, 21–30.
- Siavoshian S, Blottière HM, Le Foll E, Kaeffer B, Cherbut C & Galmiche JP (1997) Comparison of the effect of different short chain fatty acids on the growth and differentiation of human colonic carcinoma cell lines in vitro. *Cell Biology International* **21**, 281–287.
- Siavoshian S, Segain J-P, Kornprobst M, Bonnet C, Cherbut C, Galmiche J-P & Blottière HM (2000) Butyrate and trichostatin A effects on the proliferation/differentiation of human intestinal epithelial cells: induction of cyclin D3 and p21 expression. *Gut* **46**, 507–514.
- Southern E, Mir K & Shchepinov M (1999) Molecular interactions on microarrays. *Nature Genetics* **21**, 5–9.
- Steinbach G, Lynch PM, Phillips RK, Wallace MH, Hawk E, Gordon GB, Wakabayashi N, Saunders B, Shen Y, Fujimura T, Su LK & Levin B (2000) The effect of celecoxib, a cyclooxygenase-2 inhibitor, in familial adenomatous polyposis. *New England Journal of Medicine* **342**, 1946–1952.
- Steinman RA, Hoffman B, Iro A, Guillouf C, Libermann DA & el-Houseini ME (1994) Induction of p21 (WAF-1/CIP1) during differentiation. *Oncogene* **9**, 3389–3396.
- Tan S, Seow TK, Liang RC, Koh S, Lee C, Chung M & Hooi S (2002) Proteome analysis of butyrate-treated human colon cancer cells (HT29). *International Journal of Cancer* **98**, 523–531.
- Van Eyk JE (2001) Proteomics: unraveling the complexity of heart disease and striving to change cardiology. *Current Opinion in Molecular Therapeutics* **3**, 546–553.
- Wasinger VC, Cordwell SJ, Cerpa-Poljak A, Yan JX, Gooley AA, Wilkins MR, Duncan MW, Harris R, Williams KL & Humphery-Smith I (1995) Progress with gene-product mapping of the Mollicutes: *Mycoplasma genitalium*. *Electrophoresis* **16**, 1090–1094.
- Webb SJ, Nicholson D, Bubb VJ & Wyllie AH (1999) Caspase-mediated cleavage of APC results in an amino-terminal fragment with an intact armadillo repeat domain. *FASEB Journal* **13**, 339–346.
- Weinberg RA (1995) The retinoblastoma protein and cell cycle control. *Cell* **81**, 323–330.
- Whitehead RH, Young GP & Bhathal PS (1986) Effects of short chain fatty acids on a new human colon carcinoma cell line (LIM 1215). *Gut* **27**, 1457–1463.
- Wintersberger E, Mudrak I & Wintersberger U (1983) Butyrate inhibits mouse fibroblasts at a control point in the G<sub>1</sub> phase. *Journal of Cellular Biochemistry* **21**, 239–247.
- World Cancer Research Fund and the American Institute of Cancer Research (1997) *Food, Nutrition and the Prevention of Cancer: a Global Perspective*. Washington, DC: World Cancer Research Fund and the American Institute of Cancer Research.