Short-Chain Fatty Acid Modulation of Apoptosis in Gastric and Colon Cancer Cells

A thesis submitted in fulfilment of the

Doctor of Philosophy

In

The Discipline of Physiology

The University of Adelaide

By

Geoffrey Mark Matthews

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THESIS ERRATUM

Title: Short-chain fatty acid modulation of apoptosis in gastric and colon cancer cells

In response to the markers of the current thesis:

1. It would be extremely important to extend the findings within this thesis to other cancer cell lines, to animal models of gastric carcinoma, to epithelial cells and to the association between *Helicobacter pylori* infection and gastric cancer. It would also be important to consider the contribution of intestinal flora and their metabolites in the activities of SCFAs, however this was outside of the scope of this project.
2. G6PDH is the rate limiting enzyme of the oxidative pentose pathway and thus its measurement implies the overall activity of this pathway. This is explicitly described in the introduction chapter (Page 3, line 13).
3. This thesis does not have the subheading "Aims", however the specific aims of each chapter are stated in the final paragraph of each chapter introduction.
4. The lack of a consistent time response between 1-^{13}C-D-glucose oxidation and G6PDH activity does not disturb the validation of its measurement and this is discussed in chapter 2 (Page 40, line 15). We propose that both methods of measurement are required to completely appreciate the movement of glucose through the OPP and/or the TCA cycle.
5. The titles above each of the tables describing cell viability within this thesis should state that viability is measured as a percentage of total cell numbers.
6. Line 1 of page 74 should read "G6PDH activity was not altered with any concentration of butyrate".
7. Line 18 of page 134 should read: "1mM butyrate increased the percentage of TA greater than 1mM propionate".
8. Line 19 of page 193 should read: "expression of many genes, including ornithine decarboxylase (differentiation marker)".

ABBREVIATIONS

AIF	Apoptosis inducing factor
ANOVA	Analysis of variance
Apaf-1	Apoptotic protease-activating factor-1
ATCC	American type culture collection
ATP	Adenosine triphosphate
Cdx-2	Caudal related homeobox-2
CO_2	Carbon dioxide
DEM	Diethyl maleate
DHEA	Dehydroepiandrosterone
DMEM	Dulbeccos modified Eagles medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
cDNA	Complimentary DNA
dsDNA	Double-stranded DNA
DOB	Delta over baseline
DTNB	5,5'-dithiobis (2-nitrobenzoic acid)
EA	Early apoptosis
EDTA	Ethylenediaminetetraacetic acid
FACS	Flow assisted cell sorting
FBS	Foetal bovine serum
FDG-PET	2-fluoro-2deoxy-D-glucose-positron emission tomography
FITC	Fluroscein isothiocyanate
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase

G0-G1	Gap phase 0 and gap phase 1
G2-M	Gap phase 2 and mitosis
G6P	Glucose-6-phosphate
G6PDH	Glucose-6-phosphate dehydrogenase
GPX	Glutathione peroxidase
GSH	Glutathione (reduced)
GSSG	Glutathione (oxidised)
GST	Glutathione-S-transferase
Hes-1	Hairy and enhancer of split-1
IGF-I	Insulin-like growth factor I
IRMS	Isotope ratio mass spectrometry
LA	Late apoptosis
M	Molar concentration
mM	Millimolar concentration
μM	Micromolar concentration
M-MLV	Moloney murine leukaemia virus
Msi-1	Musashi-1
$NADP^+$	Nicotinamide adenine dinucleotide phosphate (oxidised)
NADPH	Nicotinamide adenine dinucleotide phosphate (reduced)
NEAA	Non-essential amino acids
NOPP	Non-oxidative pentose pathway
ODC	Ornithine decarboxylase
OPP	Oxidative pentose pathway
$^{\bullet}OH^-$	Hydroxyl radical

$^{\bullet}O_2^-$	Superoxide dismutase
PCR	Polymerase chain reaction
PTS	Phosphatidyl serine
PI	Propidium iodide
RNA	Ribonucleic acid
ROS	Reactive oxygen species
RPMI	Roswell park memorial institute
RTPCR	Reverse transcription polymerase chain reaction
S	Synthesis phase
SCFA	Short-chain fatty acid
SEM	Standard error of the mean
TA	Total apoptosis
TCA	Tricarboxylic acid cycle
TCF	Temperature correction factor
TNF	Tumour necrosis factor

ABSTRACT

Introduction: Gastric and colon cancer are major causes of mortality and morbidity worldwide. Gastric cancer is often detected at an advanced stage and current chemotherapeutics are only modestly effective against this neoplasm. Novel chemotherapeutics, chemopreventive agents and treatment strategies are required to prevent and treat gastric cancer. The ideal method to eliminate cancer cells may be the induction of apoptosis, further preventing cell proliferation and tumour growth. Recently, short-chain fatty acids (SCFAs) butyrate and propionate have been investigated as potential chemotherapeutic agents, particularly in colon cancer. Butyrate is reported to induce apoptosis in colon cancer cells and is demonstrated to modulate intracellular redox state by altering the levels of an antioxidant, glutathione (GSH). GSH availability is controlled by the oxidative pentose pathway (OPP). Very few studies have investigated the effects of butyrate on cell types other than colon cancer cells, and even less is known regarding the effects of propionate. This thesis investigated the potential for SCFAs to induce apoptosis in a gastric cancer cell line, Kato III, compared to the colon cancer cell line, Caco-2. Cell cycle regulation, OPP activity, GSH availability and glucose metabolism were also assessed. **Methods:** Initial studies developed a new technique to measure 1-^{13}C-D-glucose metabolism. Following this, Kato III and Caco-2 colon carcinoma cells were treated with butyrate or propionate (1mM, 5mM or 10mM) or a 5mM combination of both SCFAs. The induction of apoptosis and cell cycle alterations by these SCFAs were assessed using flow cytometry. OPP activity and GSH availability were assessed in both cell lines using colorimetric techniques. Butyrate metabolism was assessed using ^{13}C-butyrate. **Results:** Butyrate and propionate significantly induced apoptosis and G2-M arrest in Kato III and Caco-2 cells, although to a significantly greater extent in the latter cell line. Moreover, butyrate induced apoptosis to a significantly greater extent than propionate, in both cell lines. SCFA treatment led to the

significant up-regulation of OPP activity in both cancer cell lines while GSH availability was significantly reduced. Glucose metabolism was initially increased by all SCFA treatments, however, 72hr butyrate treatment led to its reduction. Importantly, glucose metabolism was measured using a new technique developed within this thesis. The rate of butyrate metabolism was demonstrated to correlate with the sensitivity of each cell line to this SCFA. **Conclusions:** This thesis provides evidence that SCFAs, particularly butyrate, induce apoptosis in gastric and colon cancer cells *in vitro*. The response of cancer cells to SCFAs appears complex, and involves multiple distinct mechanisms and pathways, including p53, Fas, changes to intracellular redox state and glucose metabolism. The capability of butyrate to induce apoptosis also appears to be directly related to the rate of its metabolism. Butyrate has the potential to be utilised as an adjunctive therapy for the treatment of gastric cancer and colon cancer.

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 being made available in the University Library.

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Geoffrey Mark Matthews

February 2007

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CHAPTER 1: Short-chain fatty acid modulation of apoptosis in gastric and colon cancer

1.1: Introduction

Gastric cancer and colon cancer are major causes of mortality and morbidity worldwide [1, 2]. Colon cancer remains the third most common cancer, responsible for approximately 9% of the invasive cancers that occur annually [3] whilst death due to gastric cancer alone accounts for almost 12% of cancer related mortality and 1.6% of total mortality worldwide [2]. Thus, a better understanding of the pathogenesis of these cancers and, more importantly, how they may be prevented and treated, is important.

The majority of cancers occur due to the accumulation of genetic and epigenetic changes that alter gene expression, particularly those controlling cellular proliferation [4]. Hence, the normal processes that control proliferation become overridden and allow a single cell to ultimately develop the phenotype of a tumour. It has also been proposed that tumours develop due to changes to the expression of genes that control apoptosis [1]. These changes may prevent the normal control of cell numbers by the inhibition of apoptosis, thus preventing the death of a defective cell and instead, allowing it to proliferate uncontrollably [5, 6]. The ideal method to eliminate cancer cells could, therefore, be by the induction of apoptosis thereby, preventing further cell proliferation and tumour growth.

1.2: Apoptosis

Apoptosis is an important process that eliminates unnecessary, damaged, deleterious or aged cells, and contributes to the normal homeostasis of the cell and tissues [1]. It is a tightly regulated process that is characterised by morphological changes including condensation of nuclear chromatin, compaction of cytoplasmic organelles, cell shrinkage, collapse of the mitochondrial membrane potential and changes at the cell surface, such as phosphatidylserine externalisation [7] (Figure 1.1). It is also accompanied by fragmentation of DNA into oligonucleosomal fragments with lengths usually between 180 to 200 base pairs. The key event initiating apoptosis appears to be the activation of intracellular initiator cysteine-aspartic acid proteases (caspases) by membrane bound 'death receptors' [8, 9] (Figure 1.2). These death receptors include the tumour necrosis factor (TNF) receptor, activated by TNF's, such as TNF-α, and Fas, activated by the Fas ligand, FasL [5, 10]. Activation of the initiator caspases, such as caspase-8 and caspase-10, leads to the activation of downstream caspases, such as caspase-3, that cleave essential proteins leading to cell death. The activation of caspase-3 appears to signal the inevitability of apoptosis-related morphological changes. However, the regulation of apoptosis is also under far more complex control mechanisms.

The Bcl-2 family of proteins are known to play a major role in apoptosis regulation [10-13]. The Bcl-2 proteins are localised predominantly to the nuclear membrane, endoplasmic reticulum and outer mitochondrial membrane, and are divided into two subclasses with opposing roles. Members from one subclass, such as Bcl-2 and Bcl-X_L, are anti-apoptotic and promote cell survival by inhibiting release of the mitochondrial protein, cytochrome C, from mitochondria [13, 14]. Members of the opposing subclass, such as Bax and Bik are pro-apoptotic, and thus promote cell death [13]. The activation of pro-apoptotic Bax and Bid and/or the down-regulation of Bcl-2 and Bcl-X_L can mediate apoptosis by forming pores within the mitochondrial membrane

and allow the release of pro-apoptotic factors, including cytochrome C, and apoptosis inducing factor (AIF) [13, 15]. Cytochrome C is the most important of the released factors and associates with Apaf-1 (Apoptotic protease-activating factor-1) and caspase-9 to form an apoptosome. This leads to the activation of caspase-9, another pro-apoptotic signalling protein, which culminates in the downstream activation of caspase-3 and apoptosis (Figure 1.2) [13, 16, 17].

1.3: Role of the oxidative pentose pathway, glutathione and reactive oxygen species in apoptosis

The pentose phosphate pathway is an important biochemical pathway responsible for as much as 30% of glucose metabolism in the liver, and even more in fat cells. It also provides the only *de novo* source of ribose-5-phosphate for DNA and RNA synthesis [18]. The pentose phosphate pathway consists of two arms in most organisms, the oxidative arm (OPP) and the non-oxidative arm (NOPP) (Figure 1.3). The NOPP is reversible and provides C3-C8 glycolyl units which serve as cellular assembly metabolites. Glucose-6-phosphate dehydrogenase (G6PDH) is the rate-limiting enzyme of the OPP, while both transaldolase and transketolase are thought to be rate-limiting in the NOPP. The NOPP provides 70 to 80% of ribose-5-phosphate in mammalian tissue with the remainder generated by the OPP [19]. The OPP is particularly important because of its maintenance of intracellular redox status through the provision of NADPH for the reduction of oxidised glutathione (GSSG) to reduced glutathione (GSH), an important antioxidant (Figure 1.4) [20]. The provision of NADPH for GSH synthesis is extremely important in providing protection against oxidant induced injury. Moreover, intracellular redox

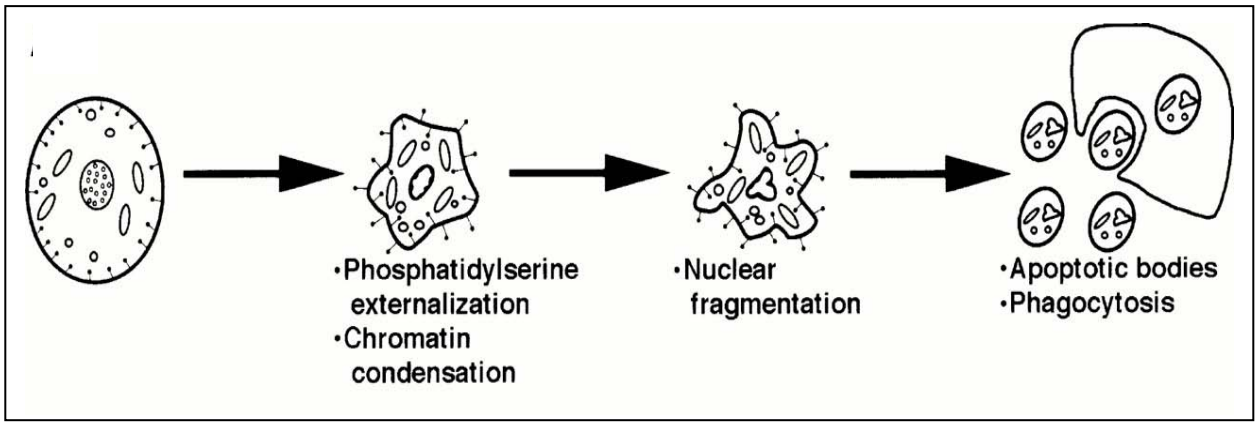

Figure 1.1: Schematic representation of the morphological features of apoptosis: A normal cell (far left) showing uniformly distributed chromatin in the nucleus with phosphatidylserine ( ) restricted to the intracellular membrane. The early morphological features of apoptosis include membrane blebbing and condensation of nuclear chromatin (second cell). Externalisation of phosphatidylserine occurs at this stage. The nucleus then becomes fragmented (third cell) and the cell separates into membrane-bound apoptotic bodies containing intact organelles (fourth cell). Apoptotic bodies are then phagocytosed by neighbouring cells.

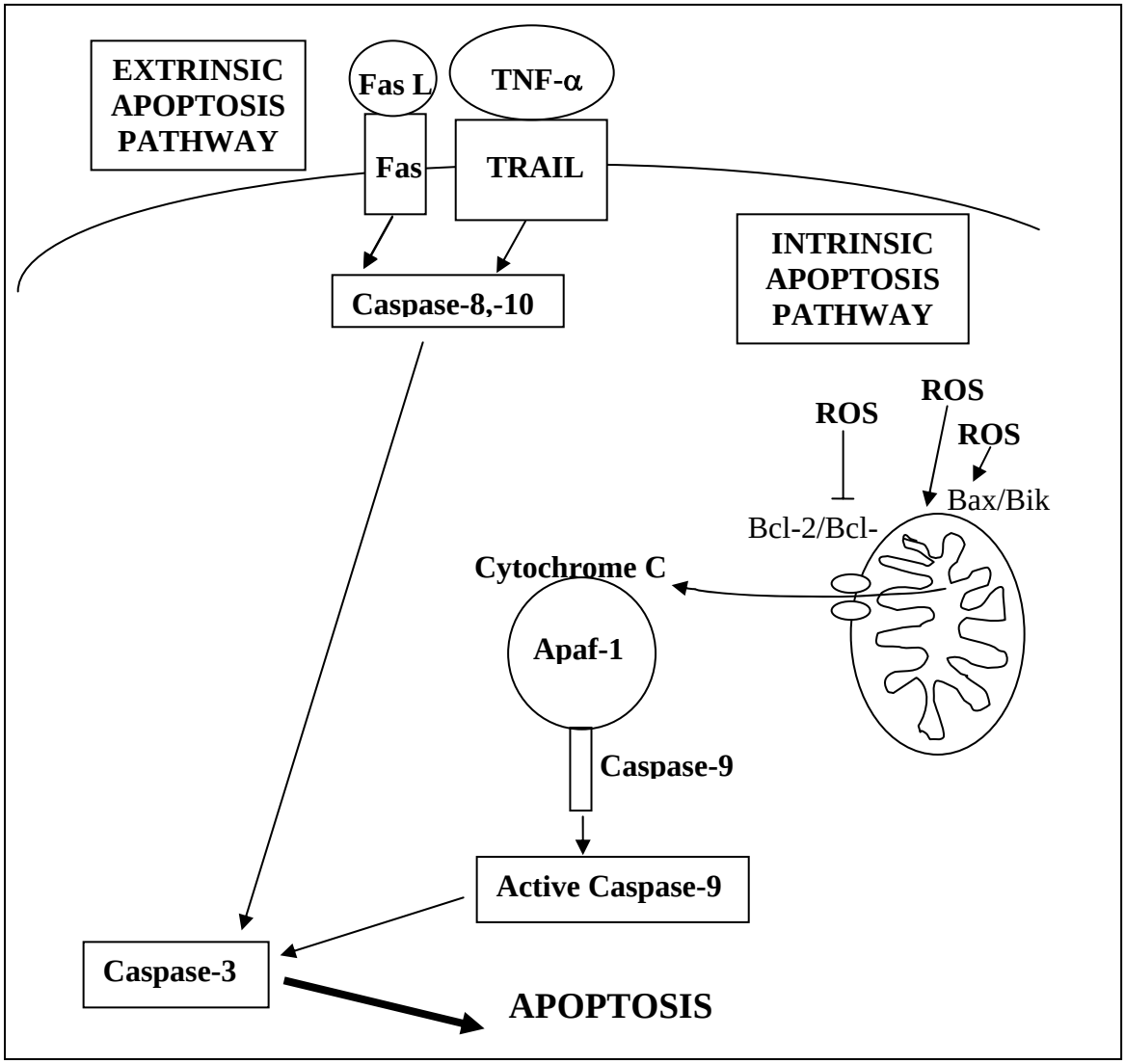

Figure 1.2: Extrinsic and intrinsic apoptosis cascades: The extrinsic cascade is activated by binding of Fas L or TNF-α to membrane bound death receptors. This activates initiator caspases-8 and -10 which directly activate down-stream caspase-3. The intrinsic cascade can be activated by intracellular ROS which induce the expression of pro-apoptotic Bax or Bid and/or decrease the expression of anti-apoptotic Bcl-2 and Bcl-X_L. This results in the formation of pores in the mitochondrial membrane allowing the release of cytochrome C which associates with Apaf-1 and caspase-9 to form an apoptosome. This leads to the activation of caspase-9 followed by the downstream activation of caspase-3 and ultimately, apoptosis.

regulation has been shown to be important in the regulation of cell proliferation and apoptosis [21, 22].

GSH plays an important role in intracellular redox regulation by controlling the reduction and inactivation of toxic reactive oxygen species (ROS) such as the hydroxyl radical ($^{\bullet}OH^{-}$) and the superoxide anion ($^{\bullet}O_2^{-}$) [23, 24]. Recent studies have proposed that ROS play an important role in the aetiology of many diseases of the gastrointestinal tract, including gastric cancer [25-27].

GSH depletion can cause cells to become sensitive to a flux of ROS [29] and high levels of cytoplasmic GSH are needed to maintain the pro-apoptotic mitochondrial protein, cytochrome C, in a reduced and inactive state [23]. Cytochrome C is located in the mitochondrial intermembrane space and is particularly sensitive to ROS [14]. Oxidative stress, due to ROS, can cause GSH levels to become depleted and allow the release and oxidation of cytochrome C from mitochondria and subsequent caspase activation. Yang and Xuesong [14] reported elevated cytochrome C levels within the cytosol and decreased levels within the mitochondria in human acute myeloid leukaemia (HL-60) cells undergoing apoptosis. These investigators also documented that apoptosis could be prevented by blocking the release of cytochrome C from mitochondria by over expressing the anti-apoptotic protein Bcl-2. Moreover, Takahashi *et al.*[30], observed that ROS-induced apoptosis was associated with inhibition of Bcl-2. Therefore, it appears that GSH plays an important role in the maintenance of cytochrome C within mitochondria by preventing ROS induced Bcl-2 inhibition.

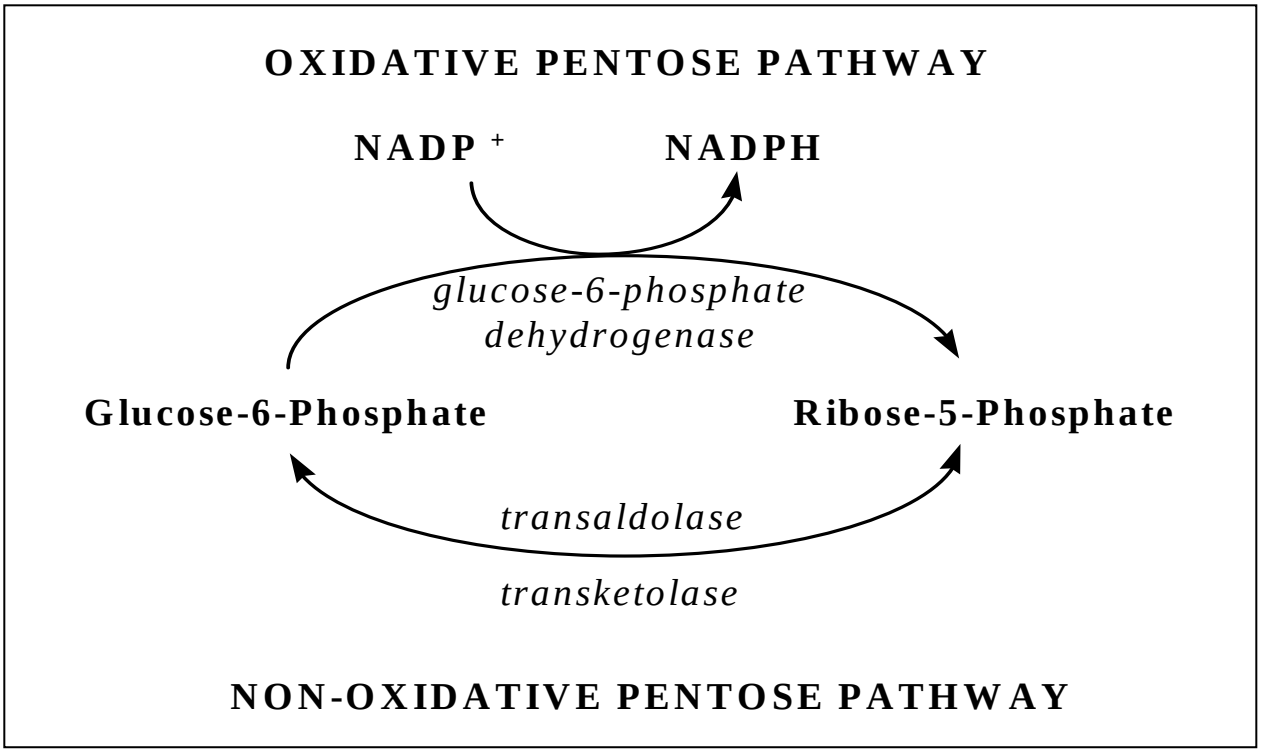

Figure 1.3: Schematic representation of the pentose phosphate pathways of glucose metabolism showing only enzymes of interest. Glucose-6-Phosphate is metabolised either through the irreversible oxidative pentose pathway or the reversible non-oxidative pentose pathway. The non-oxidative pathway can also recycle ribose-5-phosphate to glucose-6-phosphate. The rate-limiting enzyme of the oxidative pentose pathway is glucose-6-phosphate dehydrogenase (G6PDH), while both transaldolase and transketolase have been proposed as rate-limiting in the non-oxidative pathway. It has been shown that the NOPP provides approximately 70 to 80% of ribose-5-phosphate in mammalian tissue with the remainder generated by the OPP (Rais *et al.* 1999). The OPP is particularly important in that it provides the bulk of cytosolic NADPH which acts as a cofactor for a number of key antioxidant enzymes, such as superoxide dismutase and catalase. Moreover, the provision of NADPH for GSH synthesis is extremely important in maintaining intracellular redox status and providing protection against oxidant induced injury.

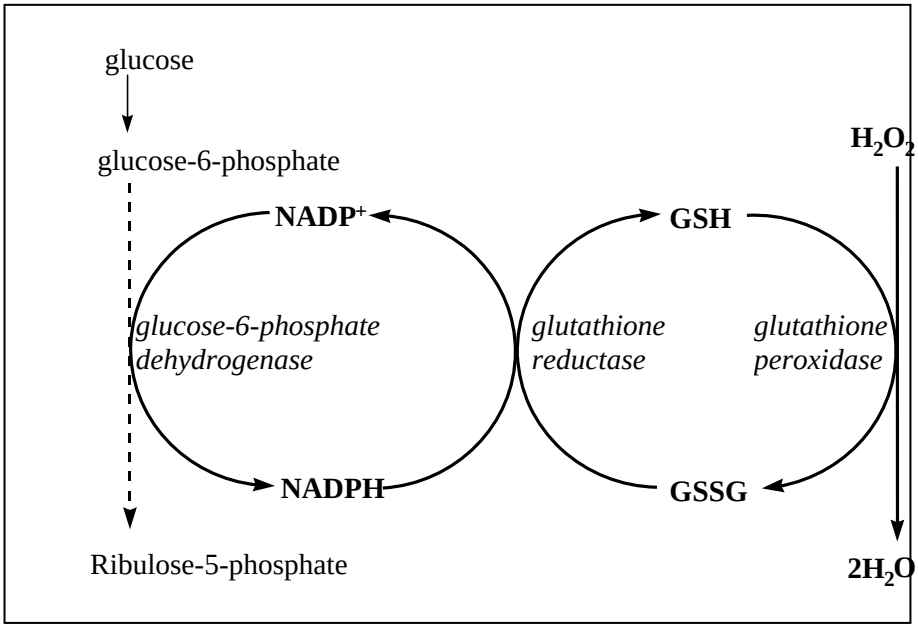

Figure 1.4: The oxidative pentose pathway provides essential NADPH for GSH synthesis. Briefly, an NADPH molecule enables the reduction of oxidised glutathione (GSSG) to reduced glutathione (GSH). GSH is an important intracellular antioxidant that has the ability to detoxify reactive oxygen species, such as hydrogen peroxide (H_20_2) [28].

Many chemotherapeutic drugs used in the treatment of cancer act by inducing apoptosis through ROS-mediated cellular damage [6, 31]. A recent study investigated whether resistance to anticancer drugs in some types of cancers may have been due to the induction of the GSH antioxidant defence system [31, 32]. Nakajima *et al.*[31] observed that elevated levels of glutathione S-transferase (GST), an enzyme of the antioxidant defence system specifically involved in the response to toxic agents such as chemotherapy, induced resistance to chemotherapy in cholangiocarcinoma. In addition, inhibition of GST led to the complete reversal of drug resistance, when combined with the chemotherapeutic agents, Adriamycin and Cisplatin, *in vitro* and a reduction in tumour volume in xenograft models of cholangiocarcinoma. Therefore, the ability of a cancer cell to sufficiently defend itself against oxidant damage due to anti-cancer agents appears largely dependent on the activity of the antioxidant defence system. Hence, inhibition of the antioxidant defence system could provide a novel mechanism to enhance apoptosis of cancer cells during anti-cancer therapy.

1.4: Short-chain fatty acids

Short-chain fatty acids (SCFAs) are produced during the anaerobic fermentation of complex carbohydrates by colonic bacteria and are found in millimolar concentrations in the lumen of the colon [11, 33]. The major SCFAs produced in the colon are butyrate, propionate and acetate, although historically, butyrate has been described as the most important [33, 34]. Roediger [33] was the first investigator to observe the uptake of butyrate by colonocytes and it is thought to be their preferred fuel source [33, 35]. Butyrate has since been shown to play an important role in maintaining colonic epithelial integrity by regulating epithelial proliferation and differentiation [36, 37]. Butyrate has also been shown to induce cell cycle inhibitors, such as cyclin D3 and $p21^{Waf1/Cip1}$ leading to cell cycle arrest in G1 phase [38, 39]. Furthermore, butyrate has been shown to induce pro-apoptotic genes *in vitro* [40, 41]. Butyrate treatment has been reported to

significantly reduce cellular protein kinase C (PKC) activity and is known to play a role in the regulation of differentiation, in two colon cancer cell lines, Caco-2 and LIM1215 [42]. More recently, Jones *et al.* [11] demonstrated that treatment of Caco-2 cells with butyrate induced apoptosis through activation of caspase-3.

It has been proposed that the ability of butyrate to induce apoptosis is dependent on the differentiation status of the colonic cell [11, 43]. Thus, it has been hypothesised that less differentiated, transformed cells are more sensitive to the apoptotic effects of butyrate than differentiated colonocytes. The molecular mechanisms that enable butyrate to induce apoptosis in transformed cells remain unclear. However, this may be primarily associated with the inability of transformed cells to properly metabolise, and hence eliminate, butyrate.

Relatively few studies have assessed the effects of butyrate on cell types other than colonic cell lines and the effects of propionate are even less well defined. Recent studies, however, have described reduced cellular proliferation and induction of apoptosis in TMK-1, SIIA and SGC-7901 gastric cancer cell lines [44-46] and also peripheral blood mononuclear T-cells, following butyrate administration [47]. Indeed, Yan and Xu [44] documented an apoptotic rate of 41.5% in SGC-7901 gastric cancer cells following treatment with 2mM Tributyrin, a pro-drug of natural butyrate. This is in contrast to treatment of colonic cells with 10mM butyrate which showed a much lower rate of apoptosis of 2.5% [11].

Potentially, this suggests that cell lines derived from sources other than the colon may have a greater sensitivity to butyrate-induced apoptosis than colonically derived cell lines. This may be due to an inability of extra-colonically derived cells to properly metabolise this SCFA. Therefore, the current thesis hypothesises that gastric cancer cells will be more sensitive to

SCFAs than colon cancer cells. Additionally, it is hypothesised that the level of SCFA-induced apoptosis will be associated with the cancer cell's ability to metabolise the SCFAs.

1.5: SCFAs and the GSH defence system

Recently, butyrate has been reported to enhance the defence of primary human colonocytes against toxic agents, by the induction of GST's which catalyse the conjugation of many electrophilic xenobiotics with GSH [48]. This enables the detoxification of harmful, potentially carcinogenic factors, and hence, may be associated with chemoprotection and prevention in colonocytes. Pool-Zobel *et al*. [48] observed an induction of many GST defence-related genes in human colon adenoma (LT97) and tumour (HT29) cells indicative of an increased defence ability. The implications of a potentially increased defence mechanism on the sensitivity of transformed cells to butyrate and/or other chemotherapeutic agents are uncertain. More importantly, induction of the GSH defence system may render colon cancer cells chemoresistant, thus increasing their probability of survival during chemotherapy. Therefore, inhibition of this defence system during chemotherapy may enhance the elimination of transformed cells.

In contrast to an up-regulation of the intracellular defence system by butyrate, other investigators have reported SCFA-induced reductions in defence related capability [49-51]. Kautenburger *et al*. [49] described a reduction in GST protein expression and stability in LT97 and HT29 cells following butyrate treatment associated with a reduction in cell viability. Moreover, it was demonstrated that the effect was significantly greater in LT97 cells than HT29 cells, and this was determined to be caused by a significantly greater uptake of butyrate by the former cell line.

Recently, Louis *et al*. [50] documented butyrate-induced GSH depletion in MCF-7 breast cancer cells that correlated with the level of toxicity induced by this SCFA. Additionally, these authors

also investigated the effects of butyrate on redox balance and other enzymes involved in cellular defence, such as glutathione peroxidase (GPX), which catalyses the reduction of ROS by GSH, and catalase, a non-GSH related enzyme that catalyses the reduction of H_2O_2. Interestingly, butyrate was initially reported to have no significant effect on cellular redox state measured by the ratio between GSH and GSSG availability. This result, however, was subsequently determined to be caused by a simultaneous decrease in both GSH and GSSG availability during butyrate treatment, and not indicative of a lack of change in redox status. Furthermore, Louis *et al*. [50] demonstrated the induction of oxidative stress by concomitant increases in GPX and catalase activation by butyrate treatment.

Therefore, debate exists in the reported effects of SCFAs, particularly butyrate, on intracellular redox status and defence. Potentially, this is due to differences in cell types and methodologies utilised within each laboratory. Hence, the current thesis hypothesises that the modulation of apoptosis in gastric compared to colon cancer cells will be associated with SCFA-induced changes to intracellular redox status and GSH availability.

1.6: Cancer, glucose metabolism and SCFAs

Glucose provides the primary carbon source for *de novo* synthesis of nucleic acids, lipids and amino acids [52] and is vital for energy metabolism and redox control in hyper-proliferative states such as cancer [53]. Glucose metabolism may be affected by changes in gene expression, culture conditions [24, 54-56] or drug treatment [52, 57-60]. However, very little is known regarding the metabolic profile of many cell types, as few simple techniques exist for its measurement.

Cancer cells are characterised by an increased level of aerobic glycolysis and an up-regulated expression of glycolytic enzymes [61, 62]. The aggressiveness of tumours has been linked to their glycolytic phenotype, inferring that up-regulated glycolytic activity must be an important component of the cancer phenotype and malignant progression [63]. Other researchers have observed that tumours rely on anaerobic pathways for the conversion of glucose to ATP [64, 65]. However, due to the low efficiency of anaerobic metabolism of glucose to lactic acid, compared to oxidation to carbon dioxide (CO_2) and water (H_2O), tumour cells maintain ATP production by increasing glucose flux. Investigations using 2-fluoro-2deoxy-D-glucose-positron emission tomography (FDG-PET) have confirmed this effect by demonstrating a significantly higher glucose uptake in primary and metastatic tumours compared to normal tissue [66, 67].

The metabolic profile of glucose flux within the major pathways of macromolecule synthesis and energy production has been assessed previously using techniques employing labelled ^{14}C or ^{13}C glucose [35, 52, 61, 68, 69]. Boren *et al*. [52] utilised the [1,2-$^{13}C_2$]glucose tracer to determine the influence of Gleevec (STI571), a potent low molecular weight protein kinase inhibitor, on glucose carbon flow in myeloid tumour cells. Gleevec treatment primarily targets metabolic enzymes, such as glucose-6-phosphate dehydrogenase (G6PDH) and hexokinase, which control glucose carbon flow through the oxidative reactions of the pentose phosphate pathway (Figure 1.4). Boren *et al*. [52] demonstrated that Gleevec altered the rate of glucose utilisation within various stages of metabolism that included nucleic acid ribose synthesis through the oxidative pentose phosphate pathway (OPP). A significant decrease in G6PDH activity was apparent following Gleevec treatment suggesting that it acted by controlling the production of reducing equivalents (NADPH) and fatty acid synthesis in the myeloid tumour cells which ultimately controls their proliferation. Therefore, the metabolic profiling of glucose metabolism in cancer cells allows a better understanding of the mechanisms by which chemotherapeutic drug

treatments can induce metabolic changes. Furthermore, metabolic profiling could be implemented to enhance the predictivity of targeted drug therapy, and determine the means by which genetic modifications can be translated into changes to cellular metabolism.

Very little is understood regarding the extent that alterations to cellular metabolism by SCFAs can affect cell growth and apoptosis. However, butyrate is reported to alter the metabolism of glucose in some cell types [38, 70, 71]. A recent study proposed that changes to intracellular redox state and gene expression by SCFAs could lead to metabolic changes that may affect cell growth [38, 71]. Indeed, Boren *et al.* [38] reported that differences existed in the metabolism of butyrate between a butyrate-sensitive (HT29) and a butyrate-resistant (MIA) cell type. They showed that, in the presence of butyrate, HT29 cells exhibited decreased glucose utilisation and readily substituted glucose with butyrate. This change in preferred substrate metabolism by HT29 cells was accompanied by increases in the expression of alkaline phosphatase, a marker of cellular differentiation. In contrast, incubation of MIA cells with butyrate had no effect on glucose utilisation and was not associated with cellular differentiation. This suggests an adaptation in the metabolic profile of butyrate sensitive cells in the presence of this SCFA that does not occur in butyrate-resistant cell types. Therefore, the current thesis aims to investigate the effects of SCFAs on D-glucose metabolism in a gastric cancer and a colon cancer cell line. Specifically, it is hypothesised that SCFA treatment will alter the metabolism of glucose in both cell lines dependent upon their capability to induce apoptosis.

1.7: Summary

In summary, gastric cancer and colon cancer are significant causes of mortality and morbidity worldwide. Recently, a change to the control of apoptosis has been proposed as a mechanism for tumour development. Therefore, previous studies have proposed that an ideal method to

eliminate cancer cells could be by the induction of apoptosis, thereby, preventing further cell proliferation and tumour growth. The OPP and the GSH antioxidant defence system could contribute significantly to a cancer cells ability to proliferate and, more importantly, resist the apoptotic effects of many anti-cancer agents. Therapeutic inhibition of the OPP and/or GSH antioxidant defence system could, therefore, increase the sensitivity of a cancer cell to the apoptotic effects of current and novel chemotherapeutic agents, such as SCFAs. It is further proposed that SCFAs may differentially-induce apoptosis in gastric and colon cancer cell lines, and that corresponding differences will exist in the metabolism of butyrate and glucose in these cell types. These studies should provide novel and exciting treatment modalities to enhance the prevention and elimination of these human neoplasms.

CHAPTER 2: A novel technique for the rapid measurement of D-glucose metabolism and oxidative pentose pathway activity in the Kato III gastric cancer cell line

2.1: Introduction

Current techniques employed to measure glucose metabolism require the growth of cells in the presence of a labelled substrate for a period of time followed by the assessment of labelled CO_2 in the growth medium [38, 52] or by the measurement of enrichment of cellular extracts [61]. In the present study we describe the development of a novel technique for the measurement of D-glucose oxidation using 1-^{13}C-D-glucose as the stable isotope tracer that is combined with the concomitant measurement of OPP activity in a gastric cancer cell line (Figure 2.1). This technique employs a novel CO_2 collection chamber that could be easily set up in any laboratory used to measure a range of metabolic parameters in many cell types (Figure 2.2). The current study validated this technique by investigating its ability to assess alterations to D-glucose metabolism and OPP activity in the Kato III gastric cancer cell line following a) serum deprivation, b) treatment with dehydroepiandrosterone (DHEA), an inhibitor of the OPP, and c) treatment with insulin-like growth factor-I (IGF-I) which is thought to increase glucose flux through glycolysis and the tricarboxylic acid (TCA) cycle. It was proposed that the assessment of D-glucose metabolism, using the novel CO_2 collection chamber, and concomitant measurement of OPP activity could provide a simple method to rapidly evaluate alterations in glucose metabolism following changes to cell culture conditions or gene expression, or following drug administration.

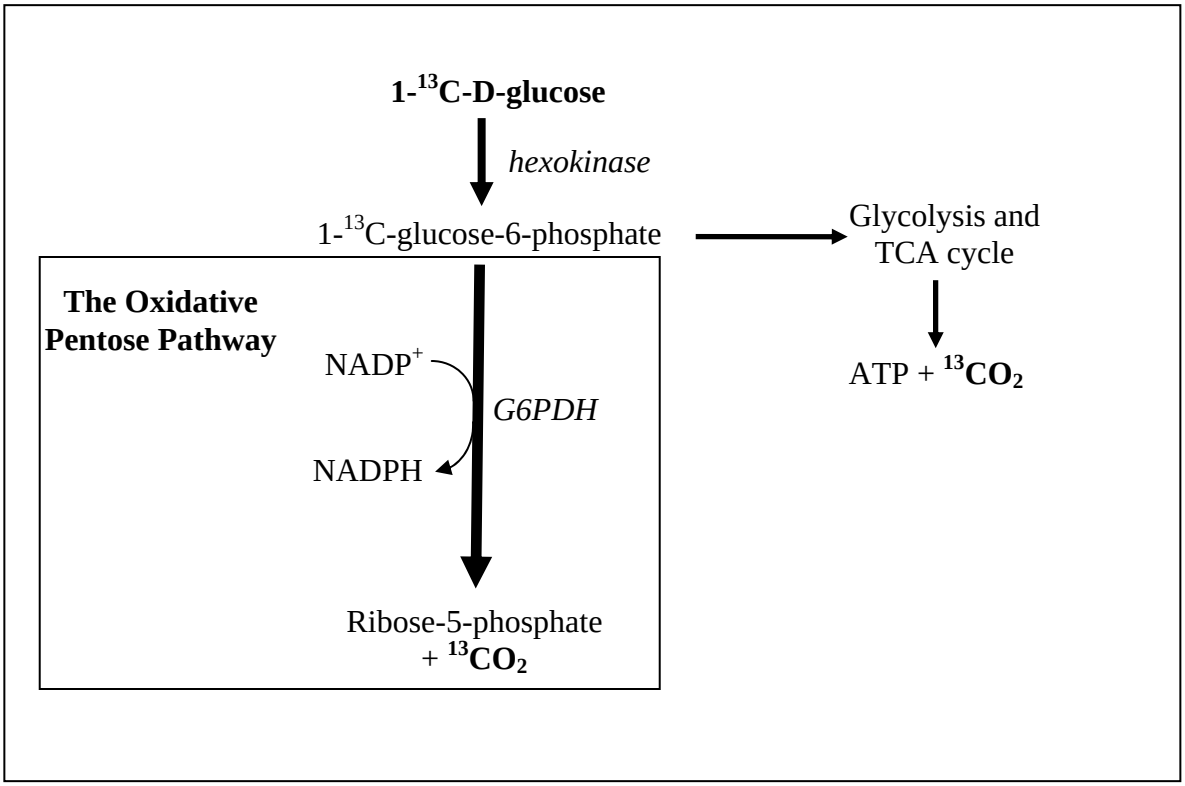

Figure 2.1: Schematic diagram of glucose metabolism through the oxidative pentose pathway (OPP), glycolysis and the TCA cycle. The first carbon of 1-^{13}C-D-glucose is preferentially metabolised by the OPP to ribose-5-phosphate and $^{13}CO_2$, however it is also oxidised within the TCA cycle during energy production.

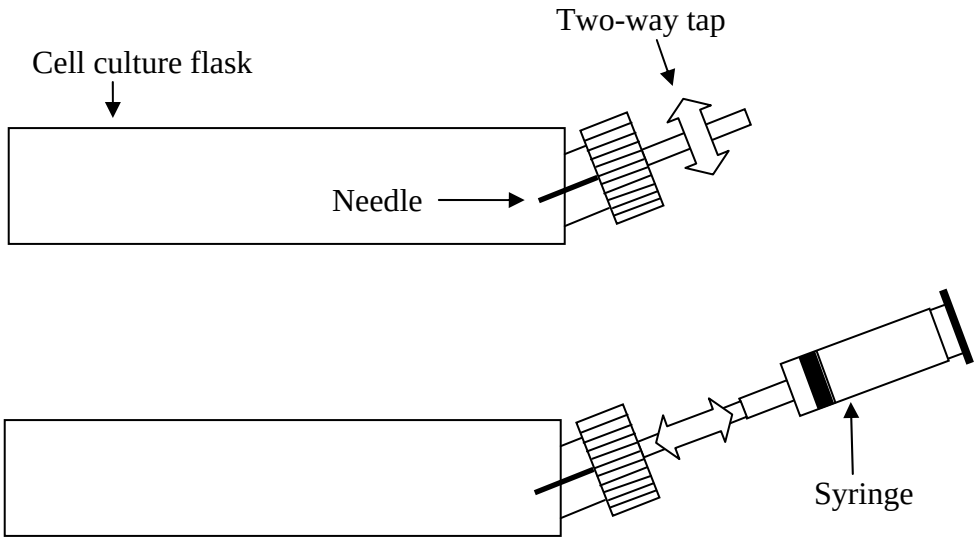

Figure 2.2: Schematic diagrams of the novel gas collection chamber. The top diagram depicts the chamber with the tap in the closed position to prevent the exchange of room air with flask air during the test. The bottom diagram shows the chamber in the gas collection/renewal state. A syringe is fitted to the needle and the tap is in the open position to allow for the collection of CO_2 from within the chamber and the addition of 5% CO_2 mixture back into the chamber.

2.2: Materials and methods

2.2.1: Materials

Kato III gastric cancer cells were obtained from American Type Culture Collection (ATCC; Manassas, VA, USA). RPMI 1640, RPMI without glucose and L-glutamine, L-glutamine, Hepes and Penicillin/Streptomycin were supplied by Invitrogen (Gibco® Australia, VIC, Australia). Heat inactivated foetal bovine serum (FBS) was obtained from JRH Biosciences (Lenexa, KS, USA). 1-^{13}C-D-glucose was obtained from Sercon Australia (Fulham Gardens, SA, Australia). Dehydroepiandrosterone (DHEA) and dimethyl sulfoxide (DMSO) were obtained from Sigma-Aldrich (Castle Hill, NSW, Australia). Stock DHEA (1mM) was prepared by dilution in DMSO. Insulin-like growth factor-I (IGF-I, GroPep Ltd, Adelaide, Australia) was donated by Assoc Prof Gordon Howarth. Cell culture flasks (non-gas-permeable; Greiner Bio-One International) were obtained from Interpath Services (West Heidelberg, VIC, Australia). The exclusion of 0.1% nigrosine was used to evaluate cell number and viability.

2.2.2: Kato III cell culture

Kato III cells were cultured in RPMI 1640 medium supplemented with 10% FBS, 4mM L-glutamine, 20mM Hepes and Penicillin/Streptomycin (73.5units/ml; 73.5µg/ml) in 75cm^2 flasks. Cells were maintained in a humidified 5% CO_2 incubator at 37°C.

2.2.3: Study protocols

2.2.3.1: Experimental set up of technique

In order to initially determine the optimal concentration of labelled glucose to utilise within these experiments, increasing doses of 1-^{13}C-D-glucose (1, 2, 3 and 4mM) were administered to Kato III cells and rates of $^{13}CO_2$ production subsequently measured. To then demonstrate the effect of

differences in cell number on $^{13}CO_2$ production, Kato III cells were seeded into cell culture flasks at $1x10^5$, $1x10^6$, $2x10^6$ and $4x10^6$ viable cells/5ml containing a final 1-^{13}C-D-glucose concentration of 2mM in RPMI without glucose. A control flask without Kato III cells, and a flask with Kato III cells but no labelled glucose, were also included.

2.2.3.2: D-glucose oxidation and OPP activity following serum deprivation

Kato III cells were removed from serum-containing medium by centrifugation (156 x g for 8min at 4°C) and resuspended in 20ml serum-free RPMI medium. Cells were assessed for OPP activity, by the assessment of the activity of its rate limiting enzyme G6PDH, and 1-^{13}C-D-glucose oxidation immediately (t = 0) and after 6hr, 12hr, 24hr and 48hr incubation at 37°C.

2.2.3.3: D-glucose oxidation and OPP activity following incubation with DHEA

To determine the effects of incubation with DHEA, a non-competitive inhibitor of G6PDH, Kato III cells were cultured in serum-free medium for 24hr prior to the addition of DHEA (in DMSO) to final concentrations of 75µM or 100µM or control (DMSO vehicle). G6PDH activity and 1-^{13}C-D-glucose oxidation was assessed in the Kato III cells after 2hr and 24hr incubation.

2.2.3.4: D-glucose oxidation and OPP activity following incubation with IGF-I

This study investigated the ability of IGF-I to alter cell viability, 1-^{13}C-D-glucose oxidation and OPP activity in 24hr serum deprived Kato III cells. Kato III cells ($1.5x10^7$ viable cells) were cultured in serum-free medium for 24hr prior to the addition of IGF-I (final concentration, 200ng/ml) or serum-free medium (control). IGF-I and control solutions were added every 24hr for a total of 72hr incubation. Kato III cells were harvested after 2hr, 24hr, 48hr and 72hr incubation and assessed immediately for G6PDH activity and 1-^{13}C-D-glucose oxidation.

2.2.4: Technique for assessment of 1-^{13}C-D-glucose oxidation

Kato III cells were removed from RPMI 1640 medium by centrifugation at 156 x g for 8min at 20°C. Cells were resuspended in glucose-free RPMI 1640 medium to give a final concentration of $5x10^6$ cells/ml.

Aliquots of cell suspension (1ml, $5x10^6$ cells) were added to 75cm^2 flasks containing 1.5ml of glucose free RPMI 1640 medium. A 1-^{13}C-D-glucose (2.5ml; 4mM) solution made up in glucose-free RPMI 1640 medium was then added to give a final concentration of 2mM D-glucose. Flasks were immediately capped and a needle was pierced through the cap and connected to a two-way tap. This provided an air tight chamber in which the milieu could be easily sampled at any time without contamination (Figure 2.2). The flasks were then partially evacuated and subsequently filled with 150ml of a 5% CO_2/air gas mix. A 10ml sample of flask air was then sampled and injected into an exetainer tube (Exetainer®, Labco, High Wycombe, England). A 5% CO_2/air mixture (10ml) was injected back into the flask to maintain a constant concentration of CO_2 within the flask (approx. 2%). Samples of flask air were collected every 15min for 2hr. Post-trial cell viability counts were then undertaken on all samples to correct the $^{13}CO_2$ production rates for differences in cell numbers. All flask air samples were analysed for change in $^{13}CO_2/^{12}CO_2$ ratio by isotope ratio mass spectrometry (IRMS; ABCA20/20 Europa Scientific). $^{13}CO_2/^{12}CO_2$ ratios were expressed as δ $^{13}CO_2$ values (‰) relative to the PeeDee Belemnite Limestone standard and changes in the δ ^{13}C level compared with baseline were expressed as δ over baseline (DOB ^{13}C). Rates of 1-^{13}C-glucose metabolism were calculated as rate of change in the DOB ^{13}C/min/$5x10^6$ viable cells by fitting a trend line to graphed DOB ^{13}C values from 15min to 120min.

2.2.5: Assay for G6PDH activity

2.2.5.1: Preparation of cytosol

Kato III cells were removed from serum-containing RPMI 1640 medium by centrifugation at 156 x g for 8min at 20°C. Cells were resuspended in a 0.25M sucrose lysis buffer (pH 7.4) containing 1mM EDTA and 10mM tris-HCl to give a final concentration of $2x10^7$ cells/ml. Aliquots (250µl) of the cell suspension containing $5x10^6$ cells, were then added to separate eppendorf tubes and set on ice for 30min to allow for complete lysis. Cell lysates were then spun at 16,060 x g for 1hr at 4°C. Cytosolic fractions were removed and set on ice prior to analysis.

2.2.5.2: G6PDH assay

G6PDH activity was assessed using a commercially available G6PDH assay kit (Trinity Biotech Plc, Bray, Co Wicklow, Ireland). Briefly, G6PDH assay reagent (500µl) containing 1.5mM NADP and 12mM maleimide, buffer, stabiliser and lysing agent was added to a 2ml cuvette. Cytosol (200µl) was then added to the cuvette, mixed and allowed to sit at room temperature for 5min to allow the sample to achieve thermal equilibrium. G6PDH assay substrate (1ml) containing 1.05mM G6P, buffer and $MgCl_2$, was then added to the cuvette. The cuvette was placed into a spectrophotometer (Perkin Elmer, Lambda 12, Uberlingen, Germany) and its absorbance read against water over a 10min period at 340nm. G6PDH activity was then determined from the following equation:

$$\textbf{G6PDH activity (U/5x10}^{\textbf{6}}\textbf{ viable cells)} = \frac{\Delta A \times 1.7 \times TCF}{0.2 \times 6.22}$$

Where:

ΔA = Rate of change in absorbance (ΔA) over the 10min

1.7 = Total reaction volume (ml)

TCF = Temperature correction factor (as per kit instructions)

0.2 = Sample volume (ml)

6.22 = Millimolar absorptivity of NADPH at 340nm

2.2.6: Statistics

All data are expressed as mean ± SEM, however, figures 1 and 2 utilise data from single experiments without replicates. Data were analysed using Student's t-tests or by one-way analysis of variance (ANOVA) with Fishers LSD *post-hoc* test. A Pearson product moment correlation was used to assess any correlation between the rate of 1-^{13}C-D-glucose oxidation and G6PDH activity. All statistics were calculated using SigmaStat 3.0 (SYSTAT Software Inc., California, USA). Significance was assumed with $p < 0.05$.

2.3: Results

2.3.1: Initial validation of technique

The rate of $^{13}CO_2$ production in Kato III cells was linear between 15min and 120min of incubation with 1-^{13}C-D-glucose (Figure 2.3). All subsequent assessments of D-glucose oxidation were determined from the rate of $^{13}CO_2$ production from 15min to 120min.

Rates of $^{13}CO_2$ production increased following incubation with 1mM, 2mM and 3mM 1-^{13}C-D-glucose reaching a maximum rate at 3mM (Figure 2.4). Therefore, all subsequent experiments utilised the mid range (2mM) concentration of glucose to prevent saturation effects.

1-^{13}C-D-glucose oxidation, assessed by $^{13}CO_2$ production, increased with increasing cell number (Figure 2.5). To allow for the measurement of the greatest differences in $^{13}CO_2$ production rates, all subsequent experiments employed $5x10^6$ viable cells per cell culture flask. No $^{13}CO_2$ was detected in samples when 1-^{13}C-D-glucose was incubated without Kato III cells or when Kato III cells were cultured without 1-^{13}C-D-glucose (data not shown).

2.3.2: Effects of serum deprivation

2.3.2.1: Kato III cell viability

The viability of Kato III cells was significantly reduced after 6hr ($p < 0.001$, $n = 4$) and 12hr ($p < 0.05$, $n = 4$) but not after 24hr ($p > 0.05$, $n = 4$) serum deprivation when compared to non-starved (0hr, $n = 7$) cells (Table 2.1). However, after 48hr serum deprivation, cell viability was again decreased to levels below that of non-starved controls ($p < 0.001$, $n = 4$).

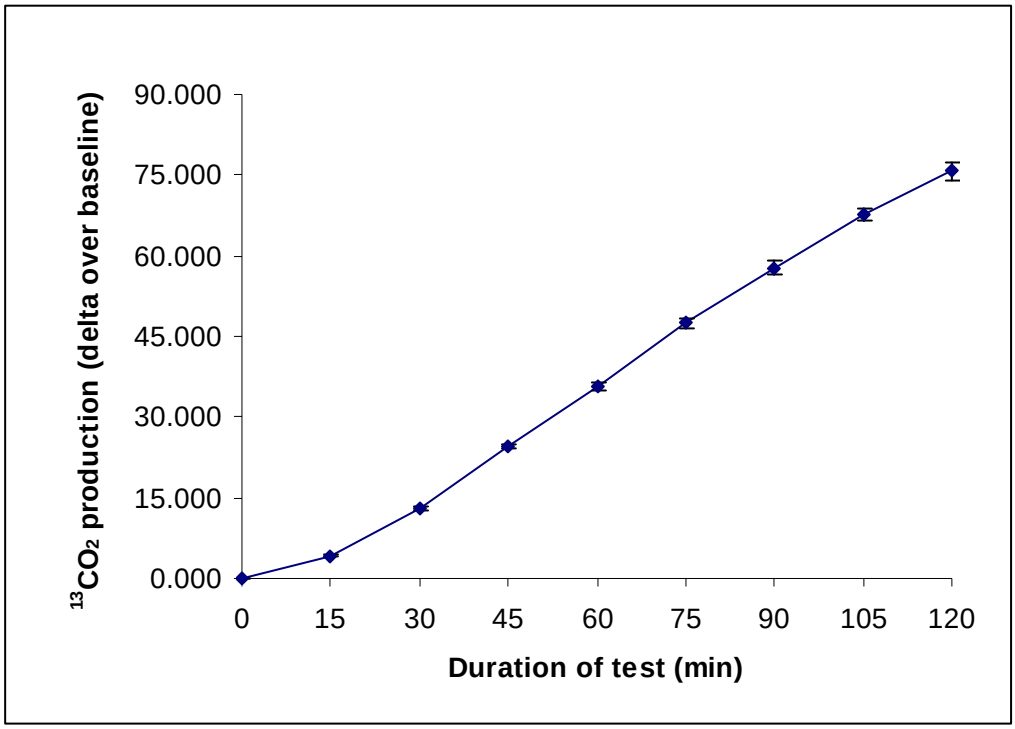

Figure 2.3: $^{13}CO_2$ production in Kato III cells following incubation of $5x10^6$ viable cells with 2mM 1-^{13}C-D-glucose. Note linearity from 15min to 120min. Data are presented as mean change from baseline (delta over baseline) ± SEM (n = 4).

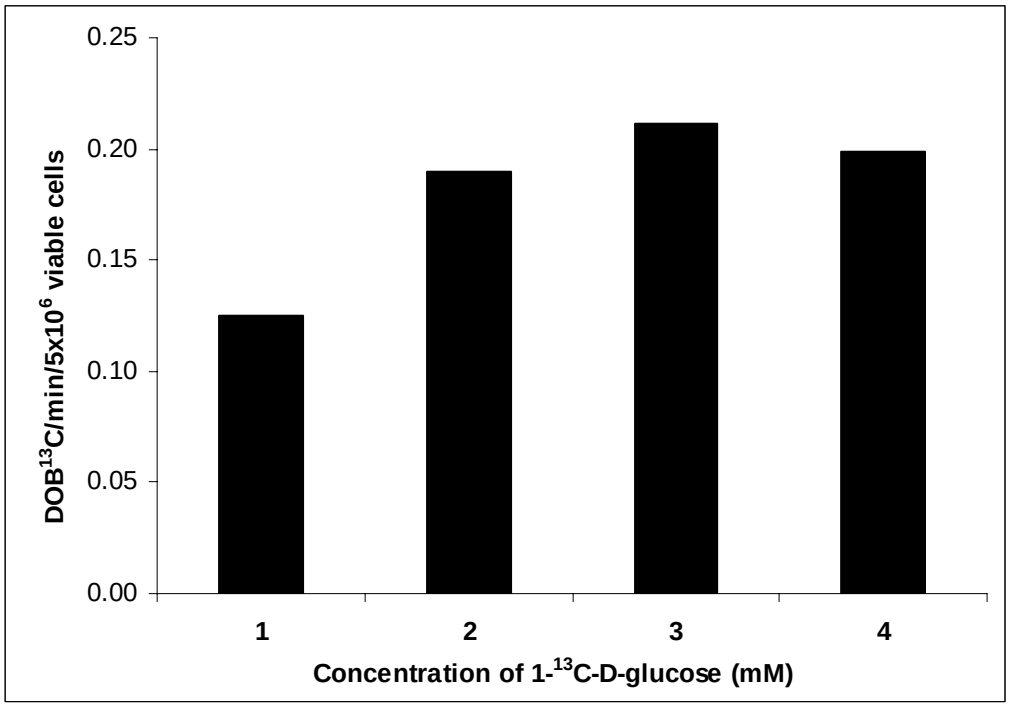

Figure 2.4: Effects of D-glucose concentration on 1-^{13}C-D-glucose oxidation in Kato III gastric cancer cells (n = 1). 1-^{13}C-D-glucose oxidation appears to increase dose responsively to a maximum at 3mM D-glucose. Accordingly, all proceeding measurements of $^{13}CO_2$ production were undertaken using 2mM D-glucose.

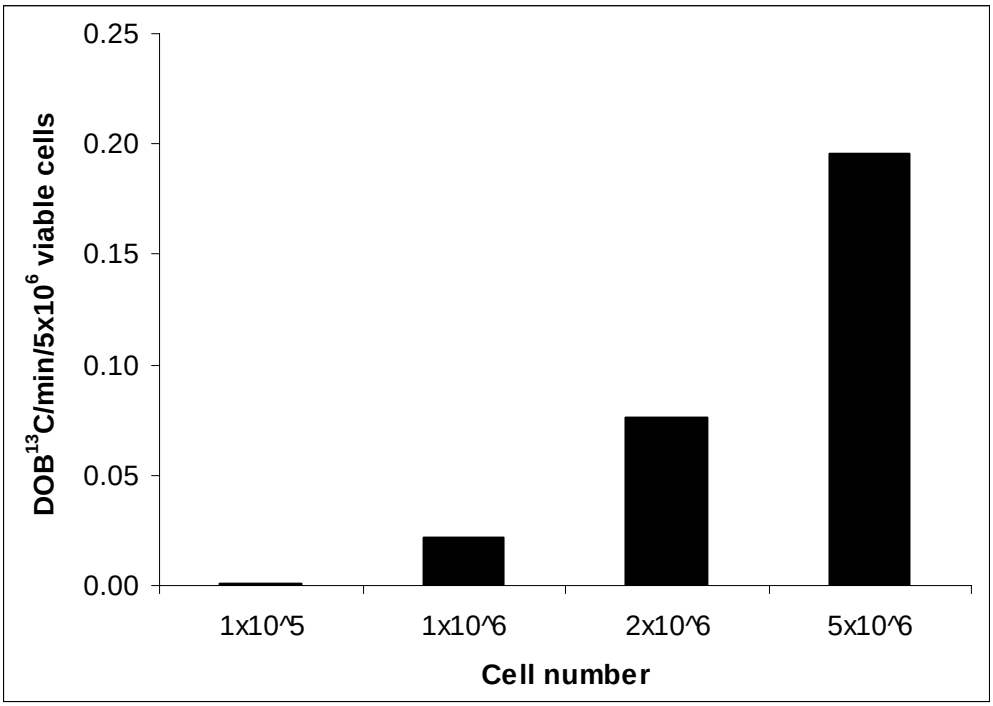

Figure 2.5: Effects of Kato III cell number on 1-^{13}C-D-glucose oxidation (n = 1). It was determined that $5x10^6$ viable cells would be utilised for all subsequent tests of D-glucose oxidation rates.

Table 2.1: Viability of Kato III cells following serum deprivation.

	Duration of treatment (hr)				
	0	**6**	**12**	**24**	**48**
Mean	96.6	84.6***	89.5*	92.0	81.2***
	(n = 7)	(n = 4)	(n = 8)	(n = 4)	(n = 4)
SEM	0.6	2.1	1.0	0.5	5.8

Kato III cell viability after 6hr, 12hr, 24hr and 48hr serum-deprivation were compared to 0hr control non-starved cells (*$p < 0.05$, ***$p < 0.001$).

2.3.2.2: G6PDH activity

Serum deprivation significantly reduced G6PDH activity after 12hr (0.059 ± 0.001, $p < 0.05$; $n = 4$) compared to the non-starved rate (0.084 ± 0.002, $n = 5$) (Figure 2.6). G6PDH activity was not significantly different to the non-starved rate after 24 (0.085 ± 0.002, $p > 0.05$, $n = 4$) and 48hr (0.078 ± 0.002, $p > 0.05$, $n = 6$).

2.3.2.3: 1-^{13}C-D-glucose oxidation

Serum deprivation reduced 1-^{13}C-D-glucose oxidation after 6hr (0.178 ± 0.060; $p < 0.001$; $n = 4$) and 12hr (0.143 ± 0.024; $p < 0.001$; $n = 8$) but not after 24hr (0.299 ± 0.0112; $p > 0.05$; $n = 4$) compared to non-starved cells (0.338 ± 0.069, $n = 7$). However, 1-^{13}C-D-glucose oxidation was significantly increased after 48hr serum deprivation (0.611 ± 0.060; $p < 0.001$; $n = 9$) compared to the non-starved rate (Figure 2.6).

It was determined that G6PDH activity correlated with 1-^{13}C-D-glucose oxidation over the first 24hr ($r = 0.88$, $p < 0.001$) period of serum deprivation but not the entire 48hr ($r = 0.4$, $p > 0.05$).

2.3.3: Effects of DHEA treatment

2.3.3.1: Kato III cell viability

The viability of Kato III cells was significantly reduced following both 2hr and 24hr treatment with 75µM (2hr, $p < 0.05$, $n = 4$; 24hr, $p < 0.001$, $n = 4$) and 100µM (2hr, $p < 0.01$, $n = 4$; 24hr, $p < 0.001$, $n = 4$) DHEA compared to control (Figure 2.7).

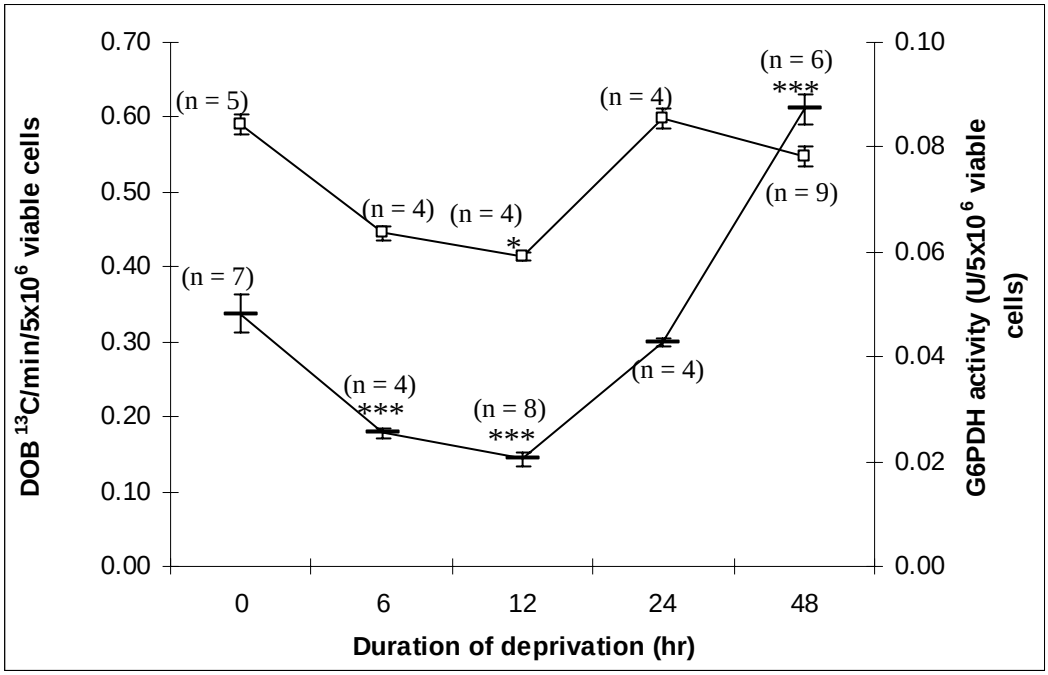

Figure 2.6: Effects of 48hr serum deprivation on 1-^{13}C-D-glucose oxidation (▬) and G6PDH activity (□) in Kato III cells. $^{13}CO_2$ production and G6PDH activity in serum-deprived cells were compared to non-starved (0hr) values. Data are presented as mean ± SEM (n = 4 – 9). *p < 0.05, ***p < 0.001.

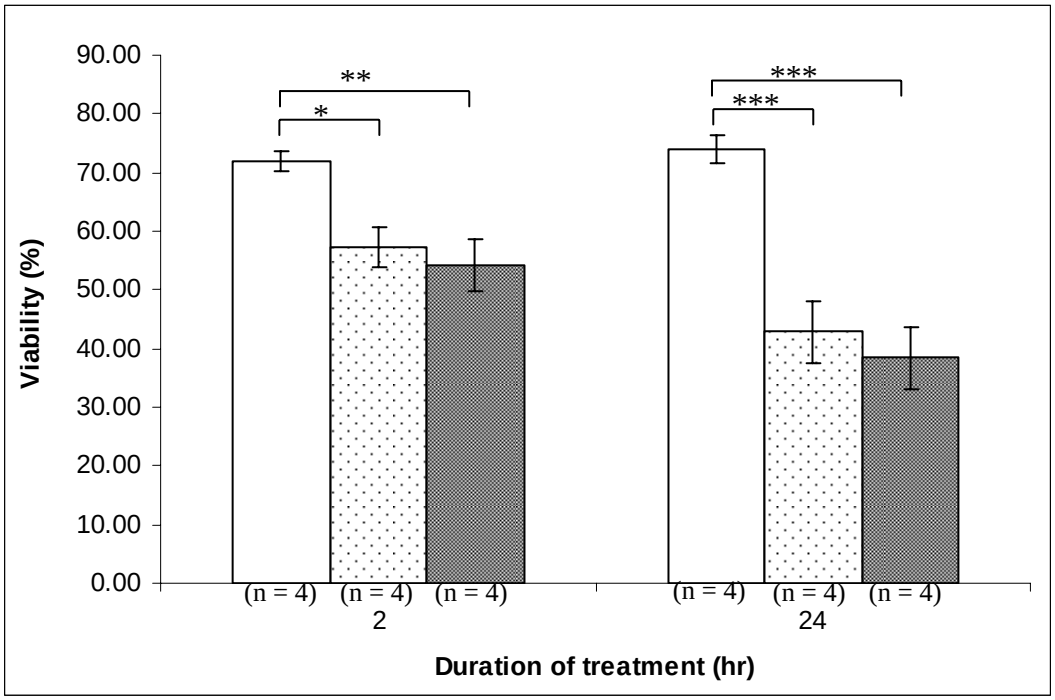

Figure 2.7: Viability of Kato III cells following incubation with 75µM or 100µM DHEA or vehicle (DMSO) control. Data are presented as mean (%) ± SEM. *p < 0.05, **p < 0.01, ***p < 0.001.

2.3.3.2: G6PDH activity

Incubation of Kato III cells with DHEA led to significant decreases in G6PDH activity after 2hr (75μM, 0.040 ± 0.001, $p < 0.001$, n = 6; 100μM; 0.023 ± 0.001, $p < 0.001$, n = 6) compared to DMSO vehicle control values (0.057 ± 0.001, n = 6) (Figure 2.8). No differences in G6PDH activity were detected after 24hr incubation with DHEA (75μM, 0.041 ± 0.002, $p > 0.05$, n = 4; 100μM; 0.036 ± 0.002, $p > 0.05$, n = 4) compared to DMSO vehicle control values (0.041 ± 0.002, $p > 0.05$, n = 6).

2.3.3.3: 1-^{13}C-D-glucose oxidation

A significant decrease in 1-^{13}C-D-glucose oxidation was observed following 2hr incubation with 100μM DHEA (0.488 ± 0.051, $p = 0.041$, n = 4) but not with 75μM DHEA (0.535 ± 0.029, $p > 0.05$, n = 4) compared to DMSO vehicle control (0.663 ± 0.048, n = 5) (Figure 2.9). No significant differences in 1-^{13}C-D-glucose oxidation were observed in Kato III cells incubated with DHEA for 24hr (75μM, 0.519 ± 0.077, $p > 0.05$, n = 5; 100μM; 0.508 ± 0.030, $p > 0.05$, n = 4) when compared to DMSO vehicle values (0.613 ± 0.085, n = 4).

2.3.4: Effects of IGF-I treatment on Kato III cells

2.3.4.1: Kato III cell viability

The percentage viability of Kato III cells was significantly decreased after 2hr incubation (26hr serum-deprivation) with IGF-I ($p < 0.01$, n = 5) compared to control (n = 4) (Table 2). Viability was significantly increased after 24hr ($p < 0.01$, n = 5, 48hr serum deprivation) and 72hr ($p < 0.001$, n = 5, 96hr serum deprivation) incubation with IGF-I but not at 48hr (60.980 ± 1.622, $p > 0.05$, n = 5, 72hr serum deprivation) compared to control values (24hr, n = 5; 48hr, n = 5; 72hr, n = 5).

2.3.4.2: G6PDH activity

G6PDH activity remained unchanged after 2hr incubation with IGF-I (0.015 ± 0.002, $p > 0.05$, n = 6) compared to vehicle control (0.018 ± 0.001, n = 6) (Figure 2.10). However, its activity was significantly decreased after 24hr (0.011 ± 0.001, $p < 0.01$, n = 5), 48hr (0.013 ± 0.001, $p < 0.05$, n = 6) and 72hr (0.012 ± 0.001, $p < 0.01$, n = 5) incubation compared to control values (24hr, 0.016 ± 0.001, n = 6; 48hr, 0.016 ± 0.001, n = 6; 72hr, 0.017 ± 0.001, n = 6).

2.3.4.3: 1-^{13}C-D-glucose oxidation

No difference in $^{13}CO_2$ production was observed after 2hr incubation with 200ng/ml IGF-I (0.387 ± 0.013, $p > 0.05$, n = 5) (Figure 2.11) when compared to 2hr control values (0.390 ± 0.023, n = 4). $^{13}CO_2$ production was significantly less than control values (24hr, 1.107 ± 0.029, n = 5; 48hr 0.935 ± 0.034, n = 5) after 24hr (0.816 ± 0.009, $p < 0.001$, n = 5) and 48hr (0.442 ± 0.011, $p < 0.001$, n = 5) incubation with IGF-I. In contrast, 72hr incubation with IGF-I significantly increased $^{13}CO_2$ production in Kato III (0.455 ± 0.022, $p < 0.01$, n = 5) cells compared to controls (0.323 ± 0.006, n = 5).

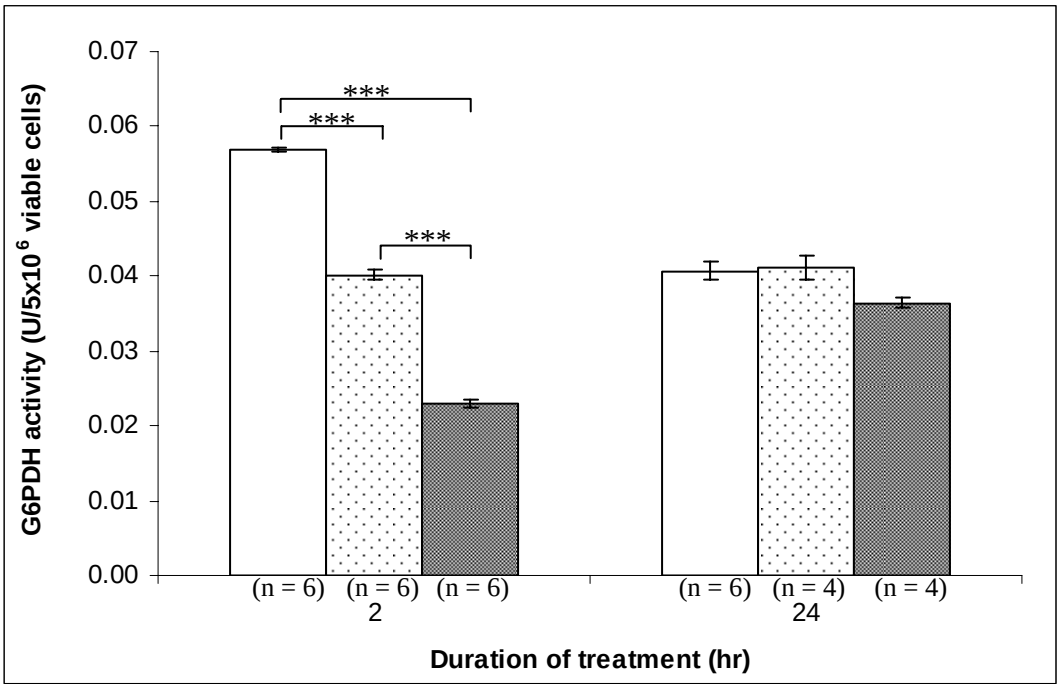

Figure 2.8: G6PDH activity in Kato III cells following incubation with 75µM ⬚ or 100µM ▩ DHEA or untreated vehicle (DMSO) control □. Data are presented as mean ± SEM. ***p < 0.001.

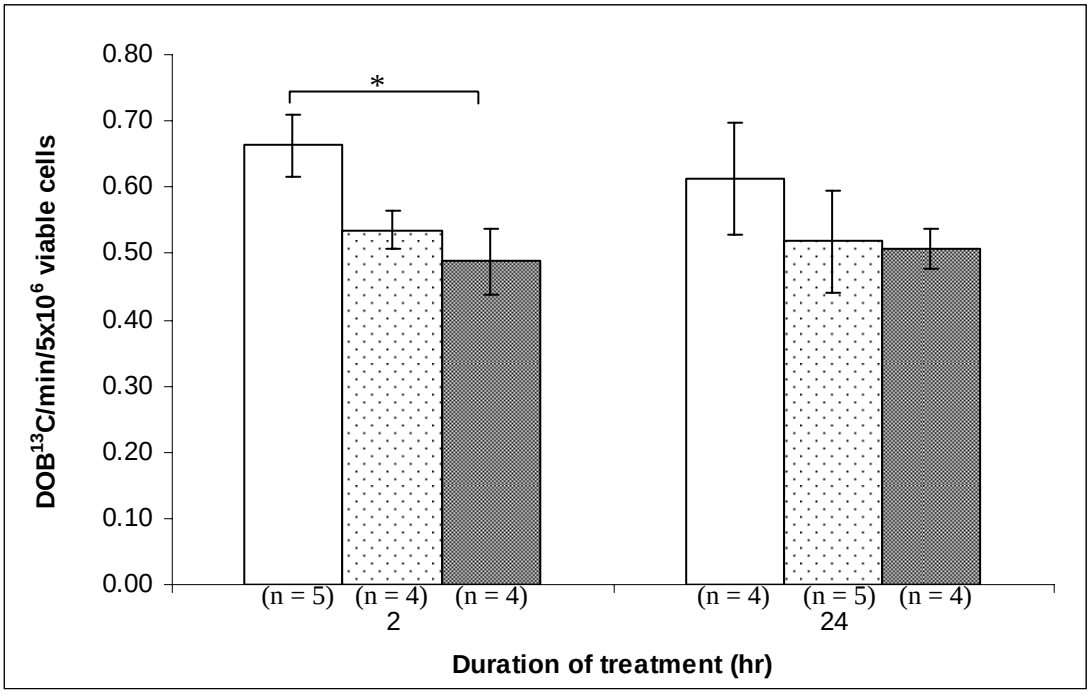

Figure 2.9: 1-^{13}C-D-glucose oxidation in Kato III cells following incubation with 75µM ⬚ or 100µM ▒ DHEA compared to untreated vehicle (DMSO) controls □. Data are presented as mean ± SEM. *$p < 0.05$.

Table 2.2: Viability (%) of Kato III cells following incubation with 200ng/ml IGF-I after 24hr serum deprivation.

	Duration of treatment (hr)			
Treatment	**2**	**24**	**48**	**72**
Control	72.3 ± 3.4 (n = 5)	66.9 ± 2.0 (n = 5)	60.1 ± 2.2 (n = 5)	35.9 ± 1.7 (n = 5)
IGF-I	65.0 ± 1.3** (n = 5)	75.9 ± 1.7** (n = 5)	61.0 ± 1.6 (n = 5)	55.2 ± 1.2*** (n = 5)

Data are presented as mean (%) ± SEM and compared to time matched control. **p < 0.01, ***p < 0.001.

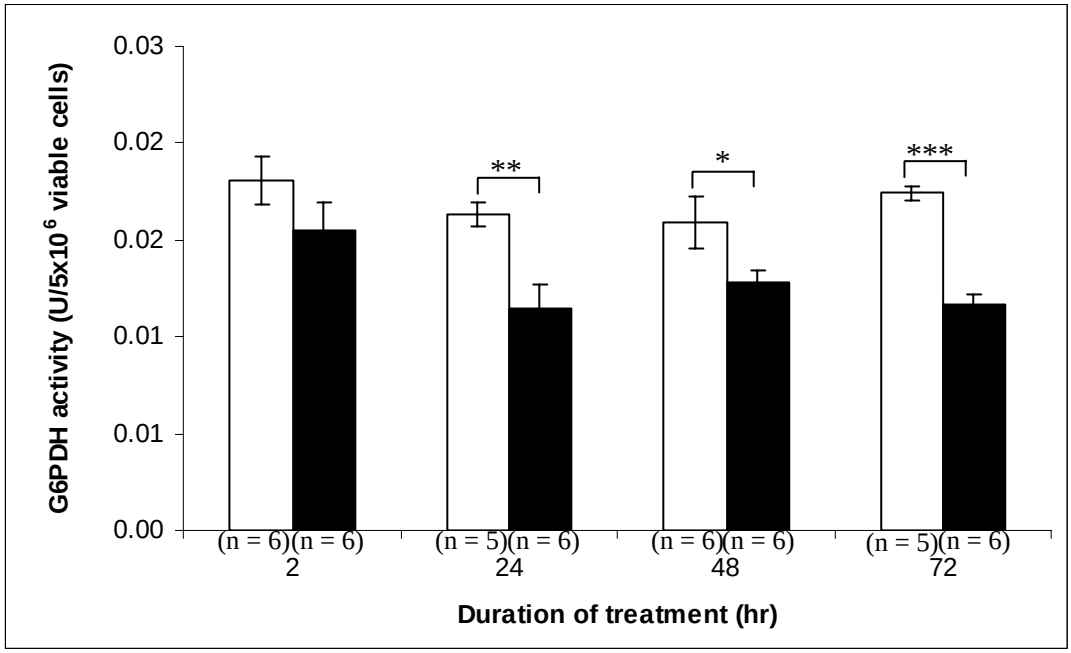

Figure 2.10: G6PDH activity in Kato III cells following incubation with 200ng/ml IGF-I ■ or vehicle control □ (serum-free medium) after 24hr serum deprivation. Data are presented as mean ± SEM. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

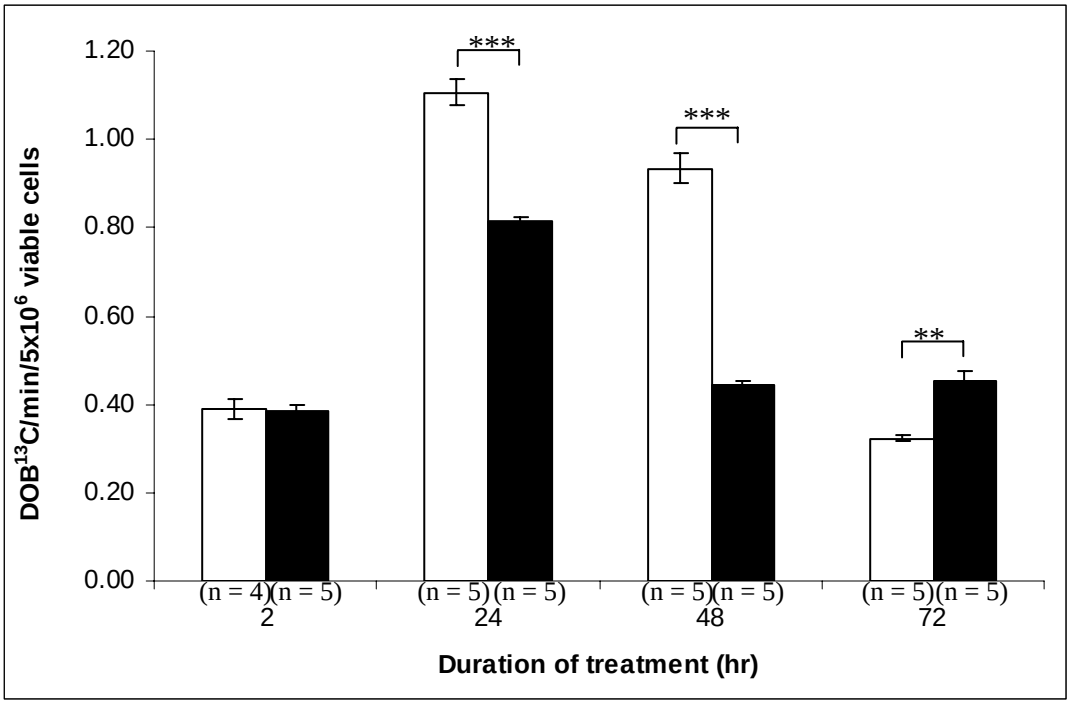

Figure 2.11: 1-^{13}C-D-glucose oxidation in Kato III cells following incubation with 200ng/ml IGF-I ■ or vehicle control □ (serum-free medium). Data are presented as mean ± SEM. **p < 0.01, ***p < 0.001.

2.4: Discussion

The present study utilised a novel CO_2 collection chamber to rapidly profile the metabolism of D-glucose in Kato III gastric cancer cells using 1-^{13}C-labelled D-glucose and the concomitant measurement of OPP activity. It was observed that serum deprivation, treatment with an inhibitor of the OPP and treatment with IGF-I significantly altered the rate of $^{13}CO_2$ production and OPP activity in this cell type.

Metabolic profiling, utilising 1-^{13}C-D-glucose as a tracer, combined with a direct measure of OPP activity allows the assessment of glucose flux within particular metabolic pathways in living cells. The basis of this new technique relies on the conversion of 1-^{13}C-D-glucose to 1-^{13}C-glucose-6-phosphate by hexokinase as it is transported into the cell and the preferential utilisation of the first labelled carbon (C-1) of glucose-6-phosphate, by the OPP. This leads to the production of unlabelled-ribose-5-phosphate and excretion of $^{13}CO_2$ (Figure 2.1). The rate of $^{13}CO_2$ production is measured using Isotope Ratio Mass Spectrometry (IRMS) and then compared to the activity of the OPP.

The combined measurement of $^{13}CO_2$ production and OPP activity provides an index of D-glucose metabolism through the OPP and/or via the tri-carboxylic acid cycle (TCA) cycle. The OPP is a pathway of glucose metabolism that provides ribose-5-phosphate for nucleic acid synthesis, and NADPH for cellular protection mechanisms [19, 22, 28, 72, 73]. The TCA cycle, however, is extremely important for cellular energy production where glucose metabolites are oxidised to ATP and CO_2 [74]. Therefore, alterations to the activity of the OPP and $^{13}CO_2$ production rate may infer differences in glucose flux between cell protection and nucleic acid synthesis or energy production.

The current study revealed a significant reduction in Kato III cell viability after 6hr and 12hr serum deprivation which was paralleled by decreases in OPP activity and 1-^{13}C-D-glucose metabolism. Unexpectedly however, by 24hr of serum deprivation, cell viability, OPP activity and $^{13}CO_2$ production had returned to non-starved levels. It is hypothesised that these serum deprived cells may initially have undergone a period of low metabolic activity to conserve energy. This may have then been followed by a period of normalised activity by 24hr to potentially increase cell protection mechanisms by fluxing glucose through the OPP while also normalising energy production. Thus, OPP activity and the rate of $^{13}CO_2$ production followed the same pattern for the first 24hr of serum deprivation.

In contrast, after 48hr of serum deprivation, the profiles of cell viability, $^{13}CO_2$ production and OPP activity were no longer identical. Serum deprivation for 48hr decreased cell viability and returned OPP activity to non-starved values, although a downward trend in OPP activity was evident. This highlights the role of the OPP in cellular growth and/or viability as reported by many other investigators [21, 22]. However, 1-^{13}C-D-glucose oxidation was significantly higher after 48hr of serum deprivation compared to non-starved cells. We propose that the rate of 1-^{13}C-D-glucose oxidation after 48hr serum deprivation did not indicate OPP activity alone, but may have signified a preferential shunting of glucose away from cell protection, and instead towards energy production.

The current study further defined the metabolic profile of Kato III cells after incubation with DHEA, an inhibitor of G6PDH. DHEA significantly decreased G6PDH activity and 1-^{13}C-D-glucose oxidation after 2hr of incubation although this effect was lost after 24hr incubation when rates returned to normal, non-treated levels. This was most likely due to the complete utilisation of DHEA by this time point. Therefore, in agreement with other studies using this inhibitor [19,

21, 22], DHEA rapidly decreased G6PDH activity and hence decreased glucose flux through the OPP.

This study also investigated the ability of IGF-I to alter cell viability, OPP activity and D-glucose oxidation in the Kato III gastric cancer cell type. Yi *et al*. [75] previously reported that IGF-I was able to stimulate the proliferation of some human gastric cancer cell lines (AGS, SNU-638). Moreover, IGF-I has been shown to induce shifts in the metabolic flux of glucose through glycolysis and the tricarboxylic acid (TCA) cycle in vascular smooth muscle cells [24] although its effects on the Kato III cell line are unknown. The current study demonstrated that IGF-I was able to alter the viability of serum-starved Kato III gastric cancer cells and that this was associated with a down-regulation of OPP activity. It appeared that IGF-I was able to partially prevent the marked up-regulation of D-glucose metabolism in the Kato III cells during prolonged serum deprivation ($\leq$ 72hr). However, this property of IGF-I was overcome after 72hr treatment (96hr serum deprivation). IGF-I, therefore, induced shifts in glucose flux in the Kato III cell line away from the OPP (cell protection) and glycolysis (energy production). This infers a reduced requirement for glucose oxidation by this cell type following IGF-I administration.

In conclusion, this study has described the development of a novel technique to rapidly measure D-glucose metabolism in Kato III gastric cancer cells. In addition, the study describes alterations to OPP activity and D-glucose metabolism in Kato III cells following a) serum deprivation, b) incubation with DHEA and c) incubation with IGF-I. This technique could provide a new tool for the assessment of glucose metabolism in a broad range of cell types, including other cancer cell lines, primary cell lines and stem cells. By profiling metabolic shifts in target cells, drug developers could identify metabolic steps that critically control cellular proliferation. It is proposed that the novel CO_2 collection chamber has the ability to be utilised for a broad range of

metabolic measures other than glucose metabolism, and in many cell types. This could aid in the future identification of novel anti-cancer agents and better define the role of changes to the expression of metabolic genes in the regulation of flux through nutrient-signalling pathways.

CHAPTER 3: Butyrate and propionate modulate apoptosis and cell cycle in Kato III gastric carcinoma cells

3.1: Introduction

Gastric cancer is the second most prevalent cause of death from cancer in the western world and is a major cause of morbidity and mortality, particularly in regions of Southeast Asia and Japan [1, 2, 76, 77]. It is often detected at an advanced stage and current chemotherapeutics are only modestly effective against this cancer [78]. Hence, novel chemotherapeutics, chemopreventive agents and treatment strategies are required to better prevent and treat gastric cancer.

Short-chain fatty acids (SCFAs), including butyrate, propionate and acetate, are produced during the anaerobic fermentation of dietary carbohydrates by colonic bacteria [11, 33, 79]. Currently, butyrate is considered to be the most important SCFA, thought to be the primary carbon source of colonocytes, and essential for maintenance of normal mucosal function [33, 70]. Butyrate has been demonstrated to induce apoptosis in colon cancer cell lines *in vitro* [41, 43] and has been proposed as an agent to treat inflammatory bowel disease [11].

Little is understood regarding the mechanisms of butyrate-induced apoptosis in cell types other than colon cancer cells and the effects of propionate are even less well-defined. SCFAs, either alone or in combination, may provide novel chemotherapeutic agents for the treatment of gastric cancer. Therefore, the present study investigated the effects of butyrate and propionate on the modulation of apoptosis and cell cycle in the Kato III gastric cancer cell line.

3.2: Materials and Methods

3.2.1: Materials

Kato III gastric cancer cells were obtained from American Type Culture Collection (ATCC; Manassas, VA, USA; ATCC#: HTB-103). RPMI 1640, heat inactivated foetal bovine serum (FBS), Glutamax, Hepes (1M) and Penicillin/Streptomycin (10,000units/ml, 10,000µg/ml) were supplied by Invitrogen (Gibco®, Invitrogen Australia, VIC, Australia). Sodium butyrate and sodium propionate were obtained from Sigma-Aldrich (Castle Hill, NSW, Australia). Stock sodium butyrate, sodium propionate and a combination of both were prepared using sterile water (200mM) and frozen at -20°C prior to the experiments. Annexin V FITC was obtained from Molecular Probes (Invitrogen Australia, VIC, Australia) and propidium iodide (PI) from Sigma-Aldrich.

3.2.2: Kato III cell culture

Kato III cells were cultured in 20ml complete RPMI 1640 medium containing 10% FBS, 4mM Glutamax, 20mM Hepes and Penicillin/Streptomycin (73.5U/ml; 73.5µg/ml) in 75cm^2 cell culture flasks. Cells were maintained at 37°C in a humidified 5% CO_2 incubator.

3.2.3: Experimental design

Kato III cells were seeded into 6 well plates ($3.5x10^6$ viable cells/well) in 5ml complete RPMI medium and incubated for 24hr at 37°C prior to treatment. Stock sodium butyrate, sodium propionate (25µl, 125µl or 250µl) or the combination of both SCFAs (125µl) was then added to the wells in duplicate to give final concentrations of 1mM, 5mM or 10mM. Control wells received 125µl sterile water. Cells were incubated at 37°C and harvested using a cell scraper

after 24hr, 48hr and 72hr. Aliquots of cell suspension (200µl) were added to flow-assisted cell sorting (FACS) tubes and assessed as described below.

3.2.4: Assays

3.2.4.1: Cell viability measurements

The differential staining of Kato III cells by Annexin V fluorescein isothiocyanate (FITC) and propidium iodide (PI) detected by flow cytometry was utilised to determine cell viability within this study. Annexin V is reported to bind to cell surface phosphatidylserine (PTS), which is externalised as an early event in apoptosis [80]. In addition, the DNA binding dye, PI, is permeable to only those cells undergoing necrosis or in the late stage of apoptosis. Hence, the combination of Annexin V and PI allows the distinction between viable, early apoptotic, late apoptotic and necrotic cells. This technique has been used widely by other investigators [16, 80, 81].

Briefly, 200µl of harvested cell suspensions (approx. $1.4x10^5$ cells) were placed into FACS tubes and immediately washed with 2ml Annexin binding buffer containing 10mM Hepes, 150mM NaCl, 5mM KCl, 1.8mM $CaCl_2$, 1mM $MgCl_2$ in 1L PBS (pH 7.4). Tubes were then centrifuged for 1min at 500 x g and supernatant was removed by inverting the tube. Annexin V FITC (2ul) plus 20µl of PI (10ug/ml) was added to each tube and then vortexed. Twenty thousand events were then collected on a FACSCalibur flow cytometer (Becton Dickinson Biosciences, San Jose, CA, USA). Results were separated into four classifications of cell viability determined by the percentage of Annexin V binding and PI staining of the Kato III cells: Total apoptotic (TA) which combined early apoptotic (EA; Annexin V only) and late apoptotic (LA; Annexin V & PI staining), necrotic (PI only) and viable cells (no Annexin V or PI). To compare differences in

viability, TA, LA, EA and necrosis in the Kato III cells following treatment with butyrate, propionate or the mixed SCFA treatment, changes from control values were calculated.

3.2.4.2: Cell cycle analysis

Cell cycle was measured by the assessment of PI binding to DNA using flow cytometry. The level of PI fluorescence is proportional to the amount of DNA present within each cell. Cells that are in G0-G1 phase have a defined amount (1x) of DNA (a diploid chromosomal DNA content). During S phase (DNA synthesis), cells contain between 1x and 2x DNA levels, whereas in G2-M phase, cells have 2x amount of DNA (tetraploid chromosomal DNA content).

Harvested cells (approx. $1.4x10^5$ cells) were removed from wells by scraping and placed into FACS tubes. A PI solution (400µl) containing 250ug/ml PI, Triton-X100 in 67% PBS in distilled H_2O was added to each tube. RNAse (DNAse free; 15µl; Sigma-Aldrich, Castle Hill, NSW, Australia) was then added to each tube to prevent PI staining of RNA. Tubes were vortexed and placed in the dark prior to analysis. Cell cycle was assessed from a minimum of 20,000 events using a FACSCalibur. Cell cycle phase was then grouped into G0-G1, S and G2-M phases.

3.2.5: Statistics

All results are presented as mean ± SEM. Data were analysed using Two-way ANOVA's with Fishers LSD *post-hoc* tests. In order to determine differences between the capability of each SCFA treatment to induce changes to cell viability, data were also transformed to express a change from control values. Statistical analyses were then undertaken on these transformed values using One-way ANOVA's with Fisher's LSD *post-hoc* tests. Statistical analysis of percentage changes in cell viability are only reported for comparisons between 5mM and 10mM

butyrate, propionate and combination treatments. All statistics were calculated using SigmaStat 3.0 (SYSTAT Software Inc., California, USA). Significance was assumed at $p < 0.05$.

3.3: Results

3.3.1: Cell viability

3.3.1.1: Butyrate decreased viability and induced apoptosis and necrosis in Kato III cells

Butyrate treatment significantly reduced the viability of Kato III cells after 24hr (5mM; $p < 0.05$, $n = 6$), 48hr (5mM, $p < 0.001$, $n = 6$; 10mM, $p < 0.05$, $n = 6$) and 72hr (1mM, $p < 0.05$, $n = 6$; 5mM, $p < 0.001$, $n = 6$; 10mM, $p < 0.001$, $n = 6$) (Table 3.1) compared to control cells. Cell viability was significantly lower at both 48hr and 72hr incubation times following 5mM butyrate treatment compared to 10mM treatment ($p < 0.05$, $n = 6$). Butyrate treatment significantly increased the percentage of total apoptosis (TA) in Kato III cells after 48hr (5mM, $p < 0.05$, $n = 6$) and 72hr (5mM, $p < 0.01$, $n = 6$) incubation compared to control but not at 1mM and 10mM ($p > 0.05$). At all concentrations tested, butyrate treatment significantly increased the percentage of Kato III cells in late apoptosis (LA) following 48hr incubation compared to non-treated controls (1mM, $p < 0.0.05$, $n = 6$; 5mM, $p < 0.001$, $n = 6$; 10mM, $p < 0.05$, $n = 6$) (Table 3.1). After 72hr treatment, the percentage of Kato III cells in LA was significantly higher than control cells when incubated with 5mM ($p < 0.001$, $n = 6$) and 10mM ($p < 0.01$, $n = 6$) butyrate. Treatment with 5mM butyrate for 48hr increased the percentage of cells in LA to a greater degree than with 10mM butyrate ($p < 0.05$, $n = 6$). The percentage of Kato III cells in early apoptosis (EA) following 24hr treatment with 10mM butyrate was significantly lower than 5mM butyrate ($p < 0.05$, $n = 6$), however, this was not significantly different to control values ($p > 0.05$, $n = 6$) (Table 3.1). Following 72hr incubation, cells treated with 1mM butyrate showed a higher percentage of EA than control cells ($p < 0.05$, $n = 6$) but not at any other concentration ($p > 0.05$).

Table 3.1: Percentage Kato III cell viability following incubation with butyrate.

Cell viability classification	Concentration of butyrate (mM)	Duration of treatment (hr) 24	48	72
Viable	**0**	66.0 ± 0.8	61.7 ± 0.7	58.3 ± 1.1
	1	62.8 ± 2.5	59.2 ± 1.6	54.3 ± 0.7[a]
	5	60.8 ± 1.9[a]	52.7 ± 1.4[cd]	43.9 ± 1.8[cd]
	10	62.1 ± 1.9	57.2 ± 1.2[ae]	48.9 ± 0.7[cde]
Total apoptotic	**0**	28.9 ± 1.4	30.1 ± 1.3	32.7 ± 1.7
	1	30.6 ± 3.6	33.6 ± 1.8	32.6 ± 1.4
	5	34.4 ± 2.3	36.1 ± 1.9[a]	41.1 ± 2.1[bd]
	10	29.7 ± 3.5	32.4 ± 1.5	36.8 ± 1.9
Late apoptotic	**0**	12.8 ± 0.4	16.3 ± 1.0	20.0 ± 1.2
	1	13.8 ± 1.9	20.3 ± 1.4[a]	22.8 ± 1.1
	5	16.4 ± 1.1	23.2 ± 1.2[c]	29.1 ± 1.7[cd]
	10	14.7 ± 1.8	20.7 ± 1.1[a]	25.1 ± 1.3[be]
Early apoptotic	**0**	16.1 ± 1.1	13.7 ± 0.4	12.7 ± 0.7
	1	16.7 ± 1.9	13.3 ± 0.5	9.8 ± 0.4[a]
	5	18.0 ± 1.3	12.9 ± 0.8	12.1 ± 0.5
	10	15.0 ± 1.8[e]	11.7 ± 0.5	11.7 ± 0.8
Necrotic	**0**	5.1 ± 0.7	8.2 ± 0.9	8.9 ± 0.9
	1	6.6 ± 1.2	7.2 ± 0.4	13.2 ± 1.2[b]
	5	4.8 ± 0.5	11.2 ± 0.6[ad]	15.0 ± 0.8[c]
	10	8.2 ± 1.8[ae]	10.4 ± 0.4[a]	14.3 ± 1.5[c]

Data are presented as mean (%) ± SEM (n = 6). All treatment groups were compared to control ([a]$p < 0.05$, [b]$p < 0.01$, [c]$p < 0.001$), 1mM butyrate ([d]$p < 0.05$) or 5mM butyrate ([e]$p < 0.05$).

In addition, the percentage of necrotic Kato III cells was significantly increased following incubation with butyrate after 24hr (10mM, $p < 0.05$, $n = 6$), 48hr (5mM, $p < 0.05$, $n = 6$; 10mM, $p < 0.05$, $n = 6$) and 72hr (1mM, $p < 0.01$, $n = 6$; 5mM, $p < 0.001$, $n = 6$; 10mM, $p < 0.001$, $n = 6$) (Table 3.1) compared to control, untreated cells ($n = 6$).

3.3.1.2: Propionate decreased cell viability and induced apoptosis in Kato III cells

Treatment of Kato III cells with propionate significantly decreased cell viability after 24hr (5mM, $p < 0.001$, $n = 6$; 10mM, $p < 0.001$, $n = 6$), 48hr (5mM, $p < 0.001$, $n = 6$; 10mM, $p < 0.001$, $n = 6$) and 72hr (5mM, $p < 0.01$, $n = 6$; 10mM, $p < 0.001$, $n = 6$) compared to un-treated control cells ($n = 6$) (Table 3.2). Propionate treatment significantly increased the percentage of TA in Kato III cells after 48hr (5mM, $p < 0.05$, $n = 6$; 10mM, $p < 0.001$, $n = 6$) (Table 3.2) and 72hr (5mM, $p < 0.05$, $n = 6$; 10mM, $p < 0.01$, $n = 6$) compared to control, un-treated values ($n = 6$). The percentage of Kato III cells in LA was significantly increased after 48hr (5mM, $p < 0.05$, $n = 6$; 10mM, $p < 0.001$, $n = 6$) (Table 3.2) and 72hr (5mM, $p < 0.001$, $n = 6$; 10mM, $p < 0.001$, $n = 6$) compared to control ($n = 6$). Propionate treatment significantly increased the percentage of cells in EA after 48hr (10mM, $p < 0.05$, $n = 6$) compared to control values. The percentage of Kato III cells undergoing necrosis significantly increased after 24hr (5mM, $p < 0.001$, $n = 6$; 10mM, $p < 0.05$, $n = 6$) but not at any other time point.

Table 3.2: Percentage Kato III cell viability following incubation with propionate.

Cell viability classification	Concentration of Propionate (mM)	Duration of treatment (hr) 24	48	72
Viable	**0**	60.7 ± 2.7	64.8 ± 0.7	58.6 ± 0.6
	1	58.1 ± 0.9	62.4 ± 1.0	59.6 ± 0.9
	5	55.6 ± 1.2[cd]	59.0 ± 0.5[c]	53.8 ± 1.3[cd]
	10	55.4 ± 1.0[cd]	57.9 ± 0.7[c]	50.7 ± 1.4[cd]
Total apoptotic	**0**	31.3 ± 2.8	20.0 ± 1.9	28.9 ± 1.1
	1	31.8 ± 1.6	23.3 ± 2.0	24.9 ± 1.4
	5	30.0 ± 1.3	25.5 ± 1.3[a]	32.4 ± 1.0[a]
	10	32.3 ± 1.7	29.5 ± 1.6[cd]	36.7 ± 1.6[cd]
Late apoptotic	**0**	14.0 ± 0.8	9.2 ± 1.1	13.9 ± 0.7
	1	15.1 ± 0.3	11.0 ± 1.1	12.2 ± 0.9
	5	14.8 ± 0.4	13.1 ± 0.9[a]	19.2 ± 0.6[cd]
	10	15.1 ± 0.3	14.6 ± 0.9[cd]	21.3 ± 1.6[cd]
Early apoptotic	**0**	17.3 ± 2.1	10.7 ± 0.8	15.0 ± 0.5
	1	16.6 ± 1.4	12.3 ± 0.9	12.6 ± 0.6
	5	15.2 ± 1.1	12.4 ± 0.5	13.3 ± 0.4
	10	17.2 ± 1.5	14.9 ± 0.8[a]	15.4 ± 0.8
Necrotic	**0**	8.0 ± 0.3	15.3 ± 1.4	12.5 ± 0.8
	1	10.2 ± 1.0	14.3 ± 1.3	15.5 ± 0.8
	5	14.3 ± 2.4[cd]	15.5 ± 1.1	15.3 ± 0.8
	10	12.2 ± 1.1[a]	12.7 ± 1.0	12.6 ± 0.8

Data are presented as mean (%) ± SEM (n = 6). All treatment groups were compared to control ([a]$p < 0.05$, [b]$p < 0.01$, [c]$p < 0.001$), 1mM ([d]$p < 0.05$) or 5mM propionate ([e]$p < 0.05$).

3.3.1.3: Butyrate/propionate combination treatment decreased cell viability and induced apoptosis in Kato III cells

Kato III viability was significantly decreased after 24hr ($p < 0.001$, $n = 6$), 48hr ($p < 0.001$, $n = 6$) and 72hr ($p < 0.001$, $n = 6$) incubation with the butyrate/propionate combination treatment compared to control values (Table 3.3). The percentage of TA in Kato III cells following treatment with the butyrate/propionate mix was significantly increased after 24hr ($p < 0.01$, $n = 6$), 48hr ($p < 0.01$, $n = 6$) and 72hr ($p < 0.001$, $n = 6$) compared to controls. Treatment with the combined butyrate/propionate solution significantly increased the percentage of LA in Kato III cells after 24hr ($p < 0.01$, $n = 6$), 48hr ($p < 0.01$, $n = 6$) and 72hr ($p < 0.01$, $n = 6$) compared to control values. The percentage of Kato III cells in EA was significantly increased after 24hr ($p < 0.001$, $n = 6$), 48hr ($p < 0.01$, $n = 6$) and 72hr ($p < 0.001$, $n = 6$) compared to control untreated cells. No significant differences in the percentage of necrotic Kato III cells were observed at any time point ($p < 0.05$, $n = 6$).

Table 3.3: Percentage viability of Kato III cells following incubation with 5mM SCFA combination treatment.

Cell viability classification	Concentration of SCFA mix (mM)	Duration of treatment (hr) 24	48	72
Viable	**0**	71.2 ± 0.6	71.0 ± 0.7	62.1 ± 0.7
	5	65.0 ± 1.1[c]	63.1 ± 0.8[c]	43.3 ± 1.0[c]
Total apoptotic	**0**	24.1 ± 1.0	16.8 ± 1.4	27.6 ± 1.7
	5	31.3 ± 1.3[c]	27.7 ± 2.1[c]	45.4 ± 2.1[c]
Late apoptotic	**0**	9.5 ± 0.4	7.8 ± 0.8	15.4 ± 1.0
	5	12.4 ± 0.7[b]	15.1 ± 1.2[b]	20.0 ± 0.8[b]
Early apoptotic	**0**	14.7 ± 0.6	9.0 ± 0.7	12.3 ± 0.7
	5	18.9 ± 0.8[c]	13.6 ± 0.8[b]	25.4 ± 1.2[c]
Necrotic	**0**	4.7 ± 0.5	12.2 ± 1.0	10.2 ± 1.1
	5	3.7 ± 0.4	8.3 ± 1.4	11.3 ± 1.0

Data are presented as mean (%) ± SEM (n = 6). All treatment groups were compared to control ($^{b}p < 0.01$, $^{c}p < 0.001$).

3.3.1.4: Butyrate/propionate combined treatment decreased viability greater than either SCFA alone

No difference in Kato III cell viability was observed between treatments after 24hr incubation (Figure 3.1). However, Kato III cell viability was significantly less following 48hr (5mM, $p < 0.05$) and 72hr (1mM, $p < 0.01$; 5mM, $p < 0.01$) treatment with butyrate compared to the same concentrations of propionate. Greater decreases in viability were obtained after 72hr incubation with the combined SCFA treatment than with butyrate (5mM, $p < 0.05$; 10mM, $p < 0.001$) or propionate (5mM, $p < 0.001$; 10mM, $p < 0.001$) alone (Figure 3.1).

The increase in TA was significantly greater following 48hr incubation with 10mM propionate than treatment with 10mM butyrate ($p < 0.01$) (Figure 3.2). The 5mM SCFA combination treatment induced TA to a significantly greater extent after 48hr than 5mM propionate ($p < 0.05$), 5mM butyrate ($p < 0.05$) and 10mM butyrate ($p < 0.01$) alone but not 10mM propionate ($p > 0.05$). A significantly higher percentage of TA was induced after 72hr treatment with 5mM butyrate than incubation with 5mM propionate ($p < 0.05$). The combination treatment induced a greater proportion of TA than 5mM butyrate ($p < 0.001$), 5mM propionate ($p < 0.001$), 10mM butyrate ($p < 0.001$) and 10mM propionate ($p < 0.001$) alone at 72hr (Figure 3.2).

The percentage of LA following 72hr incubation with 5mM butyrate was significantly greater than the 5mM ($p < 0.05$) propionate (Figure 3.3). The SCFA combination treatment induced significantly less LA than 5mM butyrate ($p < 0.05$) alone (72hr). No differences in LA were observed at any other time point.

Treatment with the combination treatment for 24hr caused greater increases in EA than following 5mM propionate ($p < 0.01$) alone (Figure 3.4). Change in the percentage of Kato III

cells undergoing EA was significantly higher after 5mM ($p < 0.05$) and 10mM ($p < 0.001$) propionate than 5mM and 10mM butyrate alone, respectively. However, 48hr combination SCFA treatment induced significantly greater increases in EA than 5mM butyrate ($p < 0.001$) and 5mM propionate ($p < 0.05$). Incubation for 72hr with the SCFA combination treatment induced EA greater than butyrate (5mM, $p < 0.001$; 10mM $p < 0.001$) and 5mM propionate (5mM, $p < 0.001$; 10mM, $p < 0.001$) (Figure 3.4).

Treatment with 5mM propionate induced a higher percentage of necrosis in Kato III cells after 24hr than 5mM butyrate ($p < 0.05$) and the combination SCFA treatment ($p < 0.05$) (Figure 3.5). However, treatment with butyrate produced a higher percentage of necrosis than propionate treatment after 48hr (5mM; $p < 0.05$, 10mM; $p < 0.01$) and 72hr (5mM; $p < 0.05$, 10mM; $p < 0.01$). The combination treatment induced significantly less necrosis after 48hr ($p < 0.001$) and 72hr ($p < 0.01$) than 5mM butyrate alone (Figure 3.5).

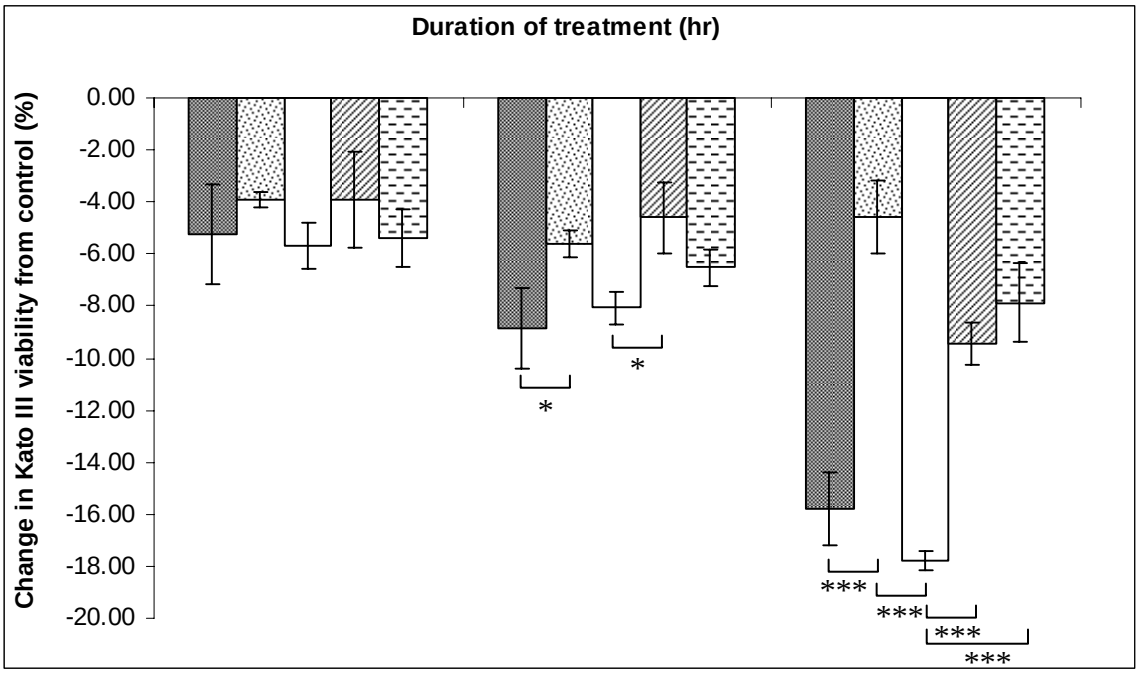

Figure 3.1: A comparison of the viability of Kato III cells following incubation with 5mM butyrate ▩, 5mM propionate ⁙ , 5mM SCFA combination □, 10mM butyrate ▨ and 10mM propionate ⁘. Data are presented as mean ± SEM (n = 6). *p < 0.05, ***p < 0.001.

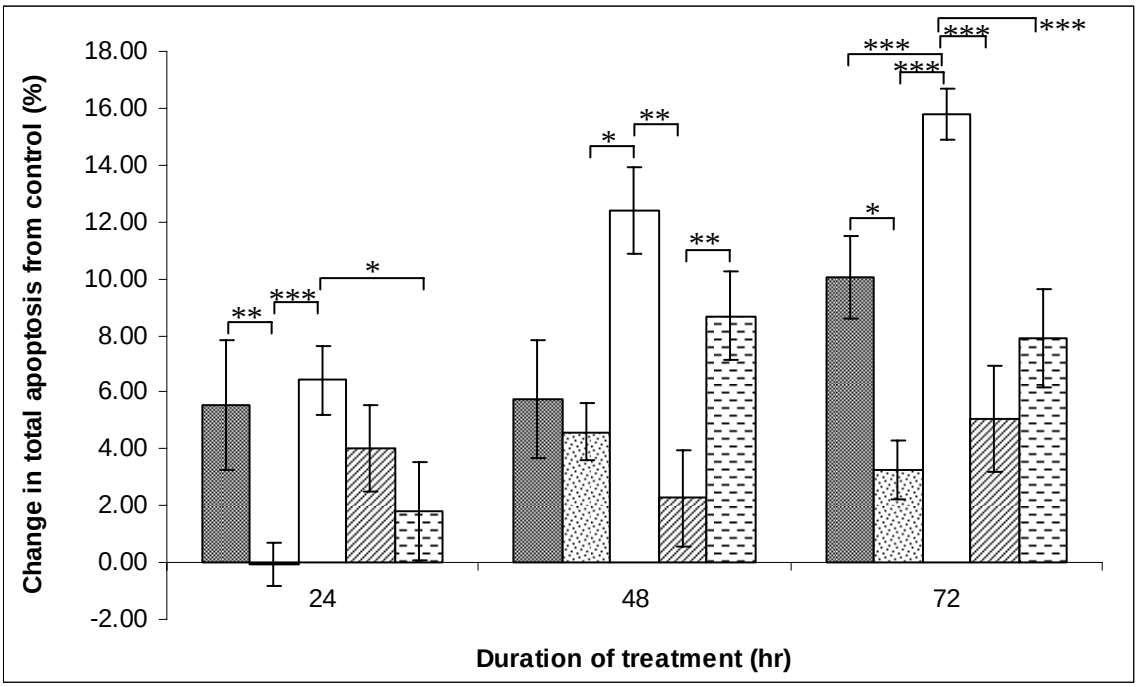

Figure 3.2: A comparison of the proportion of total apoptosis in Kato III cells following incubation with 5mM butyrate ▩, 5mM propionate ⁙, 5mM SCFA combination □, 10mM butyrate ▨ and 10mM propionate ⁝. Data are presented as mean ± SEM (n = 6). *p < 0.05, **p < 0.01, ***p < 0.001.

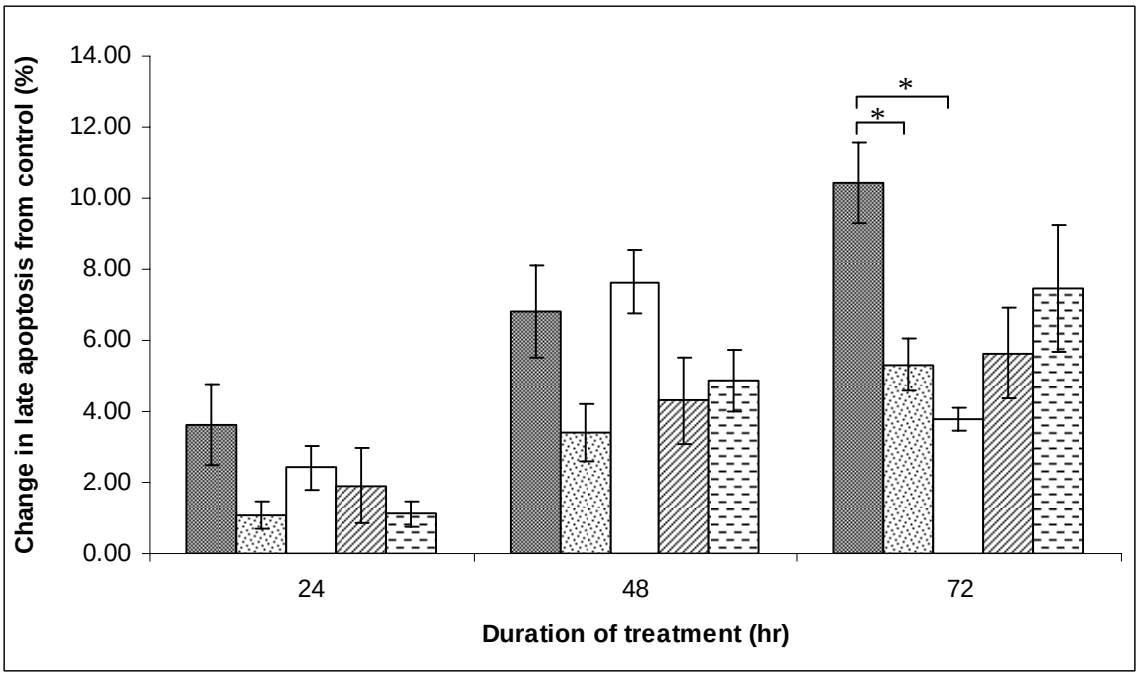

Figure 3.3: A comparison of the proportion of late apoptosis in Kato III cells following incubation with 5mM butyrate ▩, 5mM propionate ▩, 5mM SCFA combination □, 10mM butyrate ▨ and 10mM propionate ▩. Data are presented as mean ± SEM (n = 6). *$p < 0.05$.

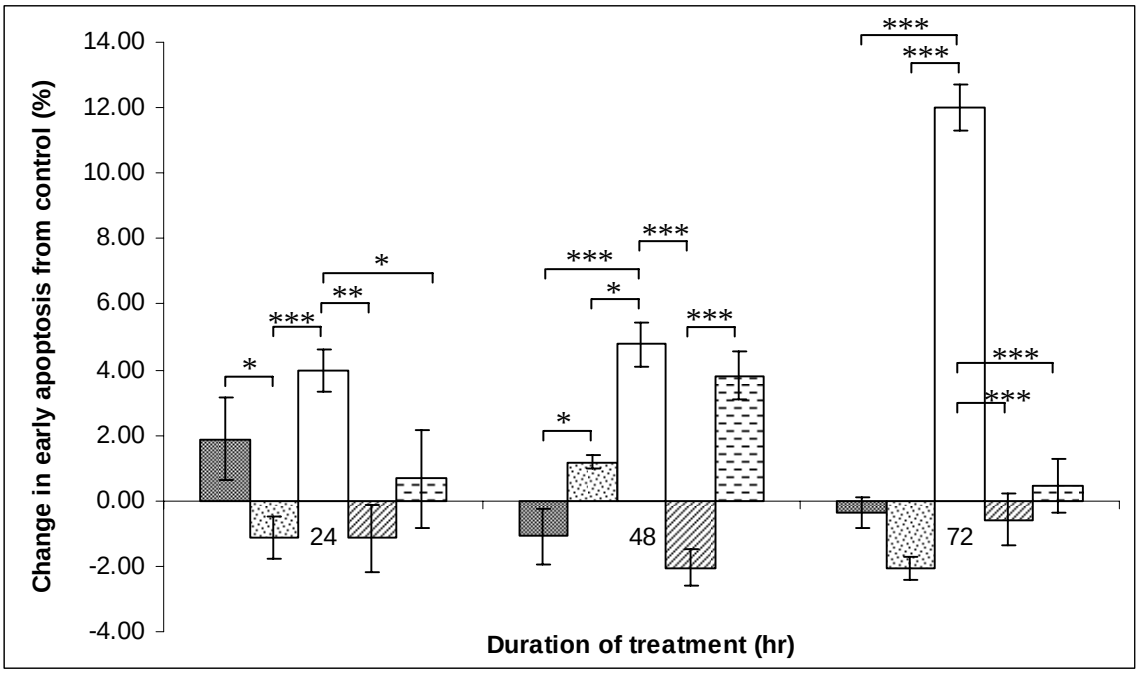

Figure 3.4: A comparison of the proportion of early apoptosis in Kato III cells following incubation with 5mM butyrate ▩, 5mM propionate ⁙, 5mM SCFA combination □, 10mM butyrate ▨ and 10mM propionate ⁝. Data are presented as mean ± SEM (n = 6). *p < 0.05, **p < 0.01, ***p < 0.001.

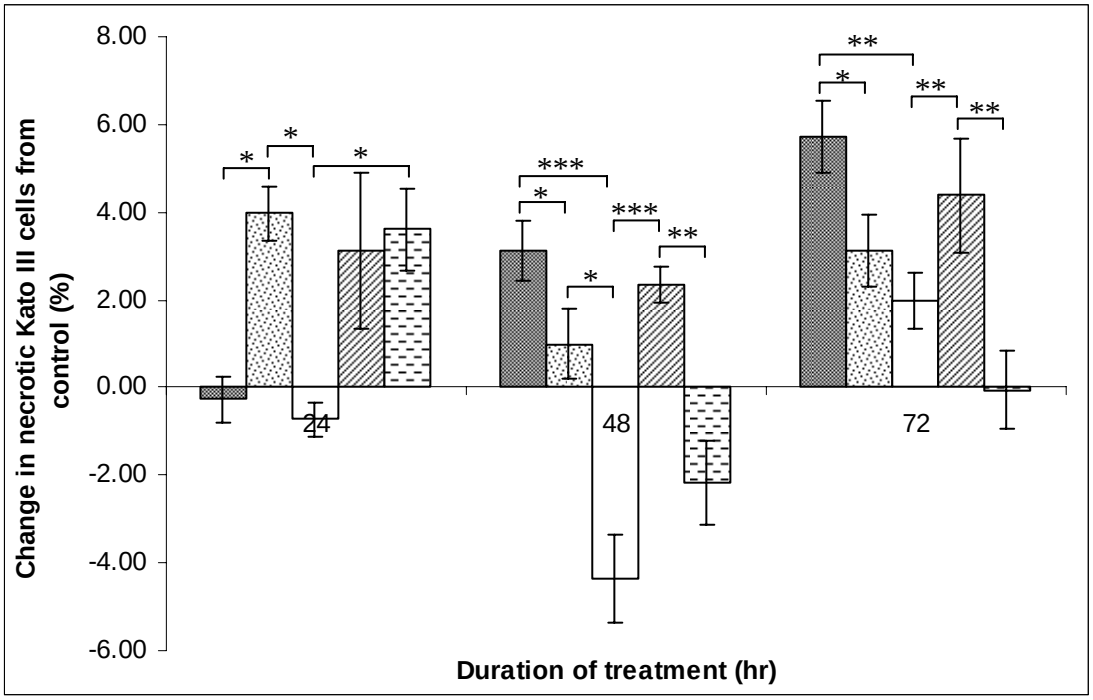

Figure 3.5: A comparison of the proportion of necrotic Kato III cells following incubation with 5mM butyrate ▩, 5mM propionate ▦, 5mM SCFA combination □, 10mM butyrate ▨ and 10mM propionate ▤. Data are presented as mean ± SEM (n = 6). *p < 0.05, **p < 0.01, ***p < 0.001.

3.3.2: Cell cycle

3.3.2.1: Butyrate induced S and G2-M phase arrest in Kato III cells

Butyrate treatment significantly decreased the percentage of Kato III cells in G0-G1 phase after 24hr (1mM, $p < 0.001$, $n = 6$; 5mM, $p < 0.001$, $n = 6$; 10mM, $p < 0.001$, $n = 6$), 48hr (1mM, $p < 0.001$, $n = 6$; 5mM, $p < 0.001$, $n = 6$; 10mM, $p < 0.001$, $n = 6$) and 72hr (1mM, $p < 0.001$, $n = 6$; 5mM, $p < 0.001$, $n = 6$; 10mM, $p < 0.001$, $n = 6$) compared to control, untreated cells ($n = 6$) (Table 3.4). The percentage of Kato III cells in S phase was significantly increased after 24hr (1mM, $p < 0.01$, $n = 6$), 48hr (1mM, $p < 0.001$, $n = 6$; 5mM, $p < 0.001$, $n = 6$; 10mM, $p < 0.001$, $n = 6$) and 72hr (5mM, $p < 0.01$, $n = 6$; 10mM, $p < 0.001$, $n = 6$) compared to control cells ($n = 6$). However, incubation with 1mM butyrate ($n = 6$) significantly reduced the percentage of Kato III cells in S phase after 72hr compared to control values ($p < 0.05$, $n = 6$). In addition, butyrate treatment significantly increased the percentage of Kato III cells in G2-M phase after 24hr (1mM, $p < 0.001$, $n = 6$; 5mM, $p < 0.001$, $n = 6$; 10mM, $p < 0.001$, $n = 6$) (Table 3.3), 48hr (1mM, $p < 0.001$, $n = 6$; 5mM, $p < 0.001$, $n = 6$; 10mM, $p < 0.001$, $n = 6$) and 72hr (1mM, $p < 0.001$, $n = 6$; 5mM, $p < 0.001$, $n = 6$; 10mM, $p < 0.001$, $n = 6$).

3.3.2.2: Propionate induced G2-M phase arrest in Kato III cells

The percentage of Kato III cells in G0-G1 phase was significantly reduced following 24hr (10mM, $p < 0.001$, $n = 6$), 48hr (5mM, $p < 0.001$, $n = 6$; 10mM, $p < 0.001$, $n = 6$) and 72hr (1mM, $p < 0.05$, $n = 6$; 5mM, $p < 0.001$, $n = 6$; 10mM, $p < 0.001$, $n= 6$) incubation with propionate (Table 3.5). Propionate treatment significantly decreased the percentage of Kato III cells in S phase after 24hr (1mM, $p < 0.01$, $n = 6$; 5mM, $p < 0.001$, $n = 6$; 10mM, $p < 0.001$, $n = 6$) and 48hr (1mM, $p < 0.05$, $n = 6$; 5mM, $p < 0.001$, $n = 6$; 10mM, $p < 0.001$, $n = 6$) but not after 72hr ($p < 0.05$, $n = 6$) compared to control values. Treatment with propionate significantly increased the percentage of cells in G2-M phase after 24hr (5mM, $p < 0.001$, $n = 6$; 10mM, $p <$

0.001, n = 6), 48hr (1mM, $p < 0.05$, n = 6; 5mM, $p < 0.001$, n = 6; 10mM, $p < 0.001$, n = 6) and 72hr (1mM, $p < 0.05$, n = 6; 5mM, $p < 0.001$, n = 6; 10mM, $p < 0.001$, n = 6) compared to untreated cells (n = 6).

Table 3.4: Cell cycle results in Kato III cells following incubation with butyrate

Cell cycle phase	Concentration of butyrate (mM)	Duration of treatment (hr) 24	48	72
G0-G1	**0**	56.4 ± 0.8	58.6 ± 1.4	59.5 ± 0.3
	1	48.7 ± 0.4[c]	51.0 ± 0.5[c]	53.8 ± 0.3[c]
	5	47.6 ± 0.8[c]	48.5 ± 0.4[cd]	51.0 ± 1.0[cd]
	10	46.0 ± 0.4[c]d	47.1 ± 1.1[cd]	48.9 ± 0.5[cde]
S	**0**	26.0 ± 0.3	19.5 ± 0.2	18.1 ± 0.4
	1	27.7 ± 0.2[b]	22.5 ± 0.3[c]	16.9 ± 0.2[a]
	5	26.7 ± 0.2	21.9 ± 0.2[c]	19.7 ± 0.6[bd]
	10	25.9 ± 0.1[d]	23.9 ± 0.7[cde]	20.4 ± 0.6[cd]
G2-M	**0**	17.4 ± 0.8	22.6 ± 1.4	21.8 ± 0.4
	1	24.1 ± 0.4[c]	26.6 ± 0.5[c]	28.5 ± 0.3[c]
	5	26.2 ± 0.8[c]	29.8 ± 0.5[cd]	27.9 ± 1.3[c]
	10	28.7 ± 0.3[cde]	29.0 ± 1.0[cd]	30.3 ± 0.3[ce]

Data are presented as mean (%) ± SEM. All treatment groups were compared to control ([a]$p < 0.05$, [b]$p < 0.01$, [c]$p < 0.001$), 1mM butyrate ([d]$p < 0.05$) or 5mM butyrate ([e]$p < 0.05$).

Table 3.5: Cell cycle results in Kato III cells following incubation with propionate

Cell cycle phase	Concentration of propionate (mM)	Duration of treatment (hr) 24	48	72
G0-G1	**0**	55.8 ± 1.2	60.5 ± 3.4	60.9 ± 0.3
	1	54.8 ± 0.3	58.0 ± 0.7	56.8 ± 0.4[a]
	5	52.5 ± 1.1	52.4 ± 1.5[cd]	52.6 ± 3.0[cd]
	10	43.6 ± 0.7[cde]	45.7 ± 0.5[cde]	45.2 ± 0.6[cde]
S	**0**	26.5 ± 1.0	17.6 ± 0.2	15.5 ± 0.1
	1	24.7 ± 0.1[b]	16.1 ± 10[a]	15.5 ± 0.2
	5	20.4 ± 0.3[cd]	13.4 ± 0.4[cd]	16.2 ± 0.3
	10	19.6 ± 0.4[cd]	13.8 ± 0.3[cd]	15.5 ± 0.4
G2-M	**0**	17.4 ± 0.5	21.7 ± 3.4	23.8 ± 0.3
	1	20.0 ± 0.3	24.8 ± 1.4[a]	27.2 ± 0.3[a]
	5	26.7 ± 1.1[cd]	33.7 ± 1.4[cd]	32.7 ± 0.7[cd]
	10	36.5 ± 1.1[cde]	40.4 ± 0.5[cde]	39.1 ± 0.4[cde]

Data are presented as mean (%) ± SEM. All treatment groups were compared to control ([a]$p < 0.05$, [b]$p < 0.01$, [c]$p < 0.001$), 1mM butyrate ([d]$p < 0.05$) or 5mM butyrate ([e]$p < 0.05$).

3.4: Discussion

The current study assessed the modulation of apoptosis and alterations to cell cycle regulation in the Kato III gastric cancer cell line by incubation with butyrate and propionate. Butyrate, propionate and the SCFA combination treatments decreased Kato III cell viability by increasing the percentage of cells undergoing apoptosis and necrosis.

Previous investigations have highlighted the apoptosis inducing effects of butyrate [41, 43], however, very few studies have assessed the effects of this SCFA on cell types other than colon cancer cells, and even fewer have investigated the effects of propionate. It was proposed, therefore, that these SCFAs would induce apoptosis in a gastric cancer cell line.

In the current study, Kato III cell viability was significantly reduced after incubation with butyrate, propionate or a 5mM combination treatment in a time-dependent manner. It was observed that butyrate treatment resulted in significantly lower cell viabilities than propionate treatment. In addition, incubation of Kato III cells with butyrate reduced cell viability to the greatest extent at a concentration of 5mM, whereas propionate treatment showed a dose responsive decrease in cell viability, resulting in the lowest cell viability at a concentration of 10mM. This is in concordance with other studies that have also reported butyrate concentrations higher than 5mM to have no added effect on cell viability [41, 82]. Additionally, the current study observed that the combination treatment, containing 5mM butyrate and 5mM propionate, induced greater decreases in cell viability than similar or higher concentrations of propionate alone, but not significantly different to butyrate alone.

The current study separated the mode of Kato III cell death into four classifications (TA, LA, EA and necrosis) dependent on the amount of Annexin V and/or PI staining detected using flow cytometry [81]. This allowed a further elucidation of the type of cell death induced by butyrate or propionate, as it has been proposed that butyrate may induce cell death by mechanisms other than by apoptosis alone [83].

It was demonstrated that butyrate induced both apoptosis and necrosis in the Kato III gastric cancer cell line at all time points (24hr, 48hr and 72hr incubation). In contrast, propionate decreased cell viability mainly by the induction of apoptosis, and caused significant death by necrosis only at the 24hr time point. Thus, butyrate appeared to endow a higher toxicity than propionate in this gastric cancer cell type, further possessing the ability to induce cell death by both apoptosis and necrosis. In addition, the combination SCFA treatment induced greater decreases in cell viability than either SCFA alone by greatly increasing the percentage of cells undergoing EA, and decreasing the number undergoing necrosis.

In the current study, the induction of apoptosis in Kato III cells following incubation with butyrate and propionate was documented to be associated with alterations to the cell cycle. Both butyrate and propionate decreased the proportion of cells in G0-G1 phase and increased the proportion of cells in G2-M phase of the cell cycle. Butyrate alone also caused significant increases in the percentage of cells in S-phase. This may have indicated arrests in S- and G2-M phases of the cell cycle in Kato III cells following SCFA treatment. Previous studies in Caco-2 cells and lymphoma cell lines (JeKo-1, Hbl-2 and Grant-519) have reported arrests in both G0-G1 and G2-M phases of the cell cycle following butyrate treatment [50, 83-85]. However, many have reported arrests in the G0-G1 [41, 44, 86] or G2-M phase alone [50, 87] in other cell types, such as breast and gastric cancer cells. Therefore, the timing of cell cycle arrest appears

dependent on the cell line used. Interestingly, the current study demonstrated cell cycle arrests in G2-M following propionate treatment, which relatively few studies have documented. This suggests that the Kato III cell type may have a unique susceptibility to propionate that is not inherent in many other cancer cell types, including colon cancer cell lines such as Caco2 and HT-29.

In conclusion, this study has documented the capacity for butyrate and propionate to induce apoptosis in a gastric cancer cell line, and has shown that this is related to G2-M phase arrest. In addition, it has shown that a treatment containing both butyrate and propionate induced similar reductions in cell viability to that obtained by butyrate alone. It is proposed that butyrate and propionate, alone or in combination, may have the potential to provide adjunctive therapies to current chemotherapeutic regimes for gastric cancer.

CHAPTER 4: Short-chain fatty acid modulation of intracellular protection and D-glucose metabolism in Kato III human gastric carcinoma cells

4.1: Introduction

Intracellular redox state is tightly controlled and is vitally important to cell survival and proper functioning [51, 88, 89]. Oxidative stress, resulting from an increase in the level of intracellular reactive oxygen species (ROS), is known to alter cellular redox state and induce apoptosis, leading to cell death [88, 90]. Changes to redox state can also alter gene expression [89], cellular metabolism and the post-translational modification of proteins [91].

Butyrate has been shown to modulate intracellular redox state by decreasing glutathione levels in human colon carcinoma (HT-29) [49, 51] and breast cancer (MCF-7) [50] cell lines. Glutathione, a cysteine-containing non-protein thiol, contains reducing and nucleophilic properties and is the major regulator of intracellular redox status in mammalian cells [89, 90, 92]. This tri-peptide exists in both a reduced (GSH) and oxidised (GSSG) form, and regulates redox state through the oxidation of its active thiol group by ROS. The synthesis of glutathione is controlled primarily by the oxidative pentose pathway (OPP) which provides NADPH for the reduction of GSSG to GSH by glutathione reductase [22, 72, 90, 93]. GSH is also reported to play a major role in resistance to chemotherapeutics in some cancers [31].

The current study investigated the potential of butyrate and propionate to modulate OPP activity, GSH availability and D-glucose metabolism in the Kato III gastric carcinoma cell line. It was

proposed that similar changes in the flux of glucose would be observed in the butyrate-sensitive Kato III cell line to that reported in HT29 cells by Boren *et al*. [38].

4.2: Materials and Methods

4.2.1: Materials

Kato III gastric cancer cells were obtained from American Type Culture Collection (ATCC; Manassas, VA, USA). RPMI 1640, heat inactivated foetal bovine serum (FBS), Glutamax, Hepes (1M) and Penicillin/Streptomycin (10,000units/ml, 10,000µg/ml) were supplied by Invitrogen (Gibco®, Invitrogen Australia, VIC, Australia). Sodium butyrate, sodium propionate, potassium phosphate, $NADP^+$, EDTA disodium salt dihydrate, glucose-6-phosphate (G6P) disodium salt, 5,5'-dithiobis (2-nitrobenzoic acid; DTNB) and glucose-6-phosphate dehydrogenase (G6PDH) were obtained from Sigma-Aldrich (Castle Hill, NSW, Australia). GSH and GSH reductase were supplied by Roche Diagnostics (Castle Hill, NSW, Australia). 1-^{13}C-D-glucose was obtained from Sercon Australia (Fulham Gardens, SA, Australia). Stock (200mM) sodium butyrate, sodium propionate and a combination of both were prepared using sterile water and frozen at -20°C prior to the experiments.

4.2.2: Kato III cell culture

Kato III cells were cultured in 20ml complete RPMI 1640 medium containing 10% FBS, 4mM Glutamax, 20mM Hepes and Penicillin/Streptomycin (73.5units/ml, 73.5ug/ml) in 75cm^2 cell culture flasks. Cells were maintained in a humidified 5% CO_2 incubator at 37°C.

4.2.3: Experimental design

In order to determine G6PDH activity and D-glucose metabolism, Kato III cells were seeded into 75cm^2 cell culture flasks ($1.5x10^7$ viable cells/flask) in 20ml medium and incubated for 24hr prior to treatment. Stock sodium butyrate, sodium propionate (100µl, 500µl or 1000µl) and the combination of both SCFAs (500µl) were added to treatment flasks to final concentrations of 1mM, 5mM and 10mM. Sterile water (500µl) was added to control flasks. Cells were incubated

at 37°C and harvested after 24hr, 48hr and 72hr incubation and immediately assessed for G6PDH activity and D-glucose metabolism.

For determination of GSH availability, Kato III cells were seeded into 6 well plates ($3.5x10^6$ viable cells/well) in complete RPMI medium (5ml) and incubated for 24hr at 37°C prior to treatment. Stock sodium butyrate or sodium propionate was then added (25µl, 125µl or 250µl) to the wells in duplicate to give final concentrations of 1, 5 or 10mM. Control cells received sterile water (125µl). Cells were incubated at 37°C and harvested after 24hr, 48hr and 72hr. For assessments of apoptosis induction and cell cycle, aliquots of cell suspension (200µl) were added to flow-assisted cell sorting (FACS) tubes and assessed as described below. For the assessment of GSH availability, all cells from duplicate wells were removed and centrifuged at 156 x g for 5min. Supernatants were removed and cells resuspended in 1ml RPMI and immediately assessed for viability and cell number by exclusion of 0.1% nigrosine before being frozen at -80°C until analysis.

4.2.4: Assays

4.2.4.1: G6PDH activity

G6PDH activity was assessed using the method described in chapter 2 (2.2.5).

4.2.4.2: GSH availability

GSH availability was assessed using a similar method to Neumann *et al*. [94]. Prior to analysis, aliquots were thawed and spun at 380 x g for 5min. Medium was removed and cells were resuspended in a Triton-X100 lysing buffer (500µl; 0.1% Triton-X100 in phosphate buffer,

10mM HCl). Next, aliquots were thoroughly mixed by vortexing to assure complete lysis. Samples were then set on ice.

Aliquots of a GSH standard (1mM) prepared in phosphate buffer (143mM, pH 7.4) were added in quadruplicate to wells of a 96 well plate and diluted in Triton X-100 lysing buffer to give final concentrations of 0.1μM, 0.2μM, 0.3μM, 0.4μM, 0.5μM, 1μM and 2μM. Triton-X100 solution (100μl) was added to blank wells. Samples (10μl) were added in quadruplicate to remaining wells and diluted in Triton X-100 lysing buffer (90μl). A phosphate buffer solution (40μl) containing 1.7mM G6P, 4.9mM DTNB, 0.17mM $NADP^+$, 5IU/ml G6PDH and 6.3mM EDTA-Na was then added to each well. Plates were incubated for 10min at room temperature prior to the addition of 5IU/ml GSH reductase (20μl) in phosphate buffer as quickly as possible using a multi-pipette. The plate was immediately set in a plate reader and the optical density measured at 405nm every 1min for 10min at room temperature. A standard curve was constructed from the reaction rates of each GSH standard and used to calculate actual GSH concentrations of samples. GSH availability was then adjusted using original cell counts (μM/1x10^6 cells).

4.2.4.3: 1-^{13}C-glucose oxidation

D-glucose oxidation was assessed in Kato III cells following incubation with 5mM butyrate, propionate and SCFA mix as described previously in Chapter 2 (2.2.4).

4.2.5: Statistics

All results are presented as mean ± SEM. Data were analysed using Student's t-tests or Two-way ANOVA's with Fishers LSD *post-hoc* tests. G6PDH activity data were also transformed to express the change in activity from control values to allow for comparisons between different

SCFA treatments (butyrate alone versus propionate alone versus butyrate/propionate mix). This was carried out by subtracting measured values for each treatment group from control values for each experiment. It must be noted that only 5mM and 10mM concentrations of each SCFA treatment were compared in this setting. These were assessed using a Kruskal-Wallis ANOVA on ranks with Student-Newman-Keuls *post-hoc* test. All statistics were calculated using SigmaStat 3.0 (SYSTAT Software Inc., California, USA). Significance was assumed at $p < 0.05$.

4.3: Results

4.3.1: Oxidative pentose pathway activity

4.3.1.1: Butyrate increased OPP activity in Kato III cells

G6PDH activity was not altered after 24hr incubation with any concentration of butyrate (Figure 4.1). However, G6PDH activity was significantly increased after 48hr incubation with butyrate at 5mM ($p < 0.001$, $n = 4$) and 10mM ($p < 0.001$, $n = 4$) compared to control (Figure 4.1). After 72hr incubation, G6PDH activity was significantly increased with 5mM ($p < 0.001$, $n = 4$) and 10mM ($p < 0.001$, $n = 4$) butyrate compared to control. At both 48hr and 72hr incubation, 10mM butyrate significantly increased G6PDH activity to a greater extent than at 5mM ($p < 0.001$) and 1mM ($p < 0.001$).

4.3.1.2: Propionate increased OPP activity in Kato III cells

Treatment with propionate significantly increased G6PDH activity in Kato III cells after 24hr (10mM, $p < 0.001$, $n = 4$), 48hr (5mM, $p < 0.001$, $n = 4$; 10mM, $p < 0.001$, $n = 4$) and 72hr (5mM, $p < 0.001$, $n = 4$; 10mM, $p < 0.001$, $n = 4$) compared to untreated controls (Figure 4.2). In addition, 10mM propionate induced a significantly greater increase in G6PDH activity than both 1mM and 5mM propionate at all time points ($p < 0.01$). Interestingly, although both butyrate and propionate induced increases in G6PDH activity in this cell line, the greatest increase in activity after 72hr incubation with 10mM butyrate ($0.61U/5x10^6$ viable cells) was almost 10-fold higher than that achieved after 72hr incubation with 10mM propionate ($0.07U/5x10^6$ viable cells).

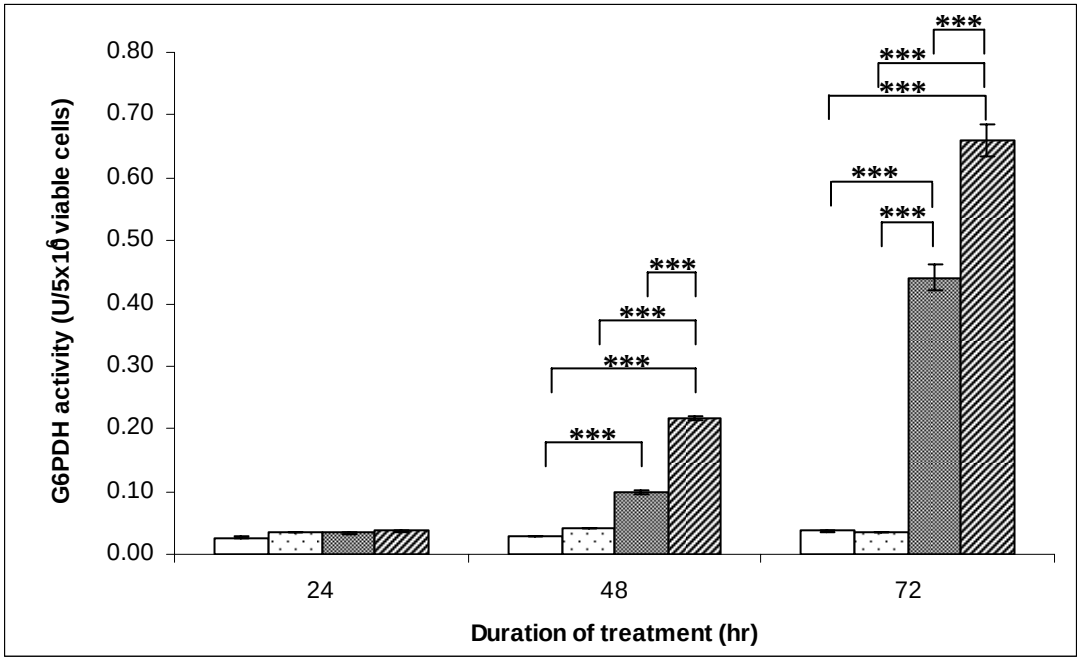

Figure 4.1: OPP activity in Kato III cells following incubation with 1mM , 5mM , and 10mM butyrate compared to vehicle control . Data are presented as mean ± SEM (n = 4). **p < 0.01, ***p < 0.001.

4.3.2.3: Butyrate/propionate combination increased OPP activity in Kato III cells

G6PDH activity was significantly increased after 24hr ($p < 0.05$, $n = 4$), 48hr ($p < 0.05$, $n = 4$) and 72hr ($p < 0.001$, $n = 4$) incubation with 5mM butyrate/propionate combination treatment than vehicle control, untreated cells (Figure 4.3).

4.3.2.4: Butyrate alone increased OPP activity greater than propionate alone and butyrate/propionate combination treatment

At all time points, butyrate treatment induced greater increases in G6PDH activity than propionate alone (1mM, $p < 0.05$; 5mM, $p < 0.05$; 10mM $p < 0.05$) (Figure 4.4). Additionally, 24hr incubation with 5mM butyrate/propionate mix induced greater increases in G6PDH activity than butyrate (5mM, $p < 0.05$; 10mM, $p < 0.05$) and propionate (5mM, $p < 0.05$; 10mM, $p < 0.05$) alone. However, increases in G6PDH activity after 48hr and 72hr were significantly less in those cells treated with 5mM butyrate/propionate mix than butyrate alone (5mM, $p < 0.05$; 10mM, $p < 0.05$) but consistently greater than after propionate treatment alone (5mM, $p < 0.05$; 10mM, $p < 0.05$) (Figure 4.4).

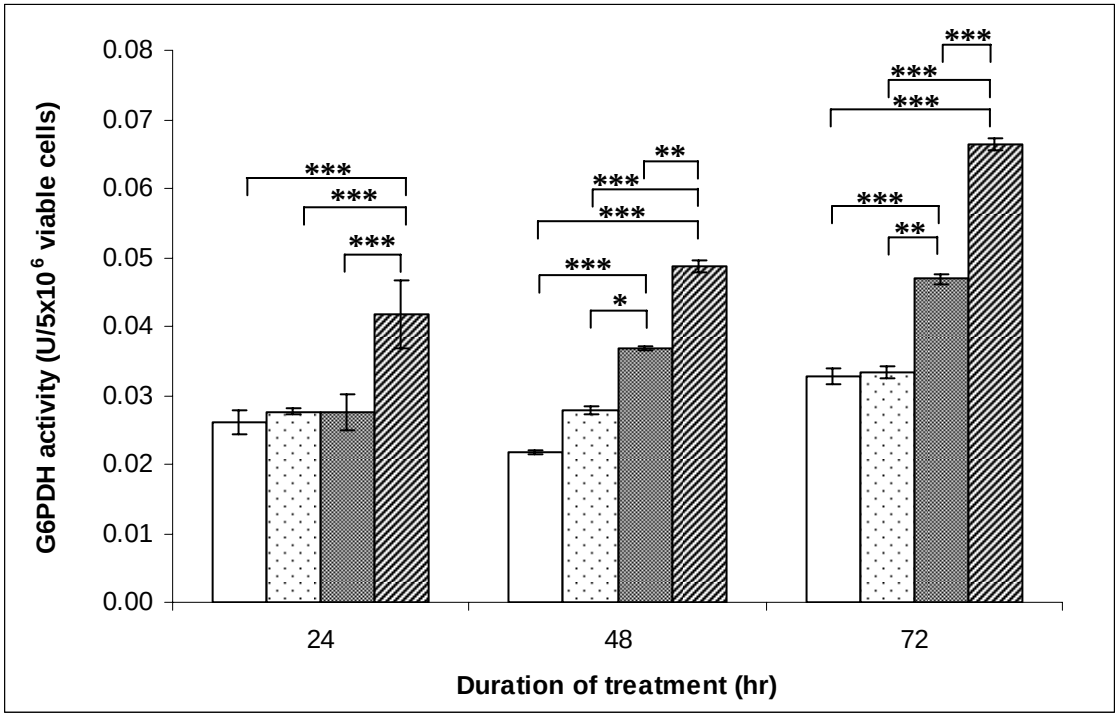

Figure 4.2: OPP activity in Kato III cells following incubation with 1mM ⬚, 5mM ▒ and 10mM ▨ propionate compared to vehicle control □. Data are presented as mean ± SEM (n = 4). *p < 0.05, **p < 0.01, ***p < 0.001.

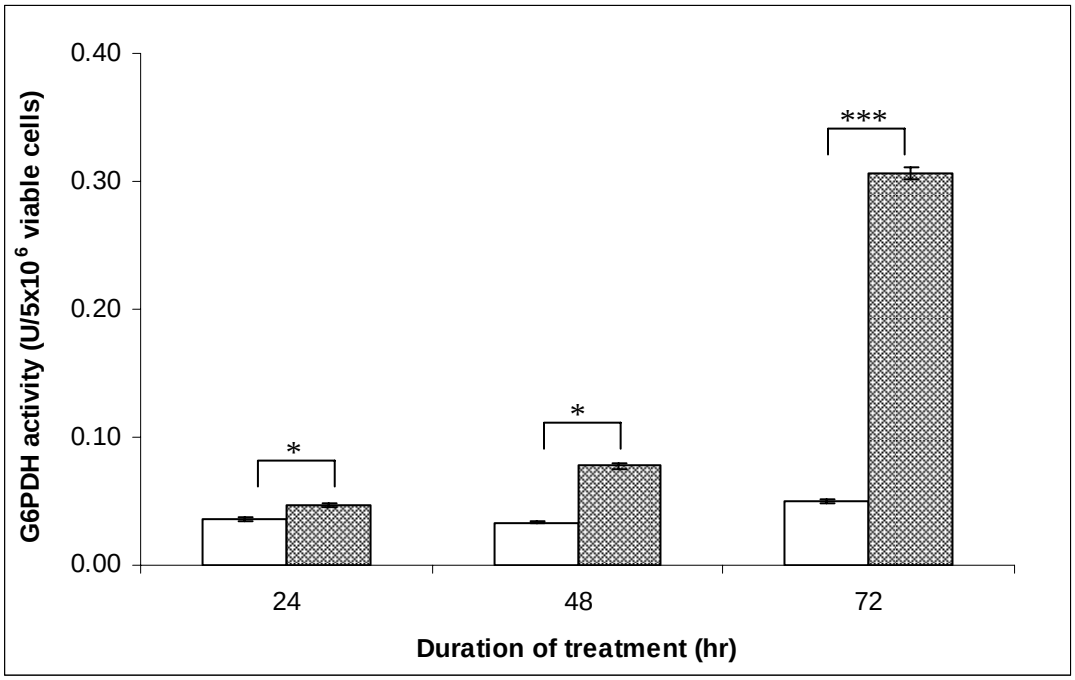

Figure 4.3: OPP activity in Kato III cells following incubation with 5mM SCFA combination treatment ▨ or vehicle control □ . Data are presented as mean ± SEM (n -= 4). *p < 0.05, ***p < 0.001.

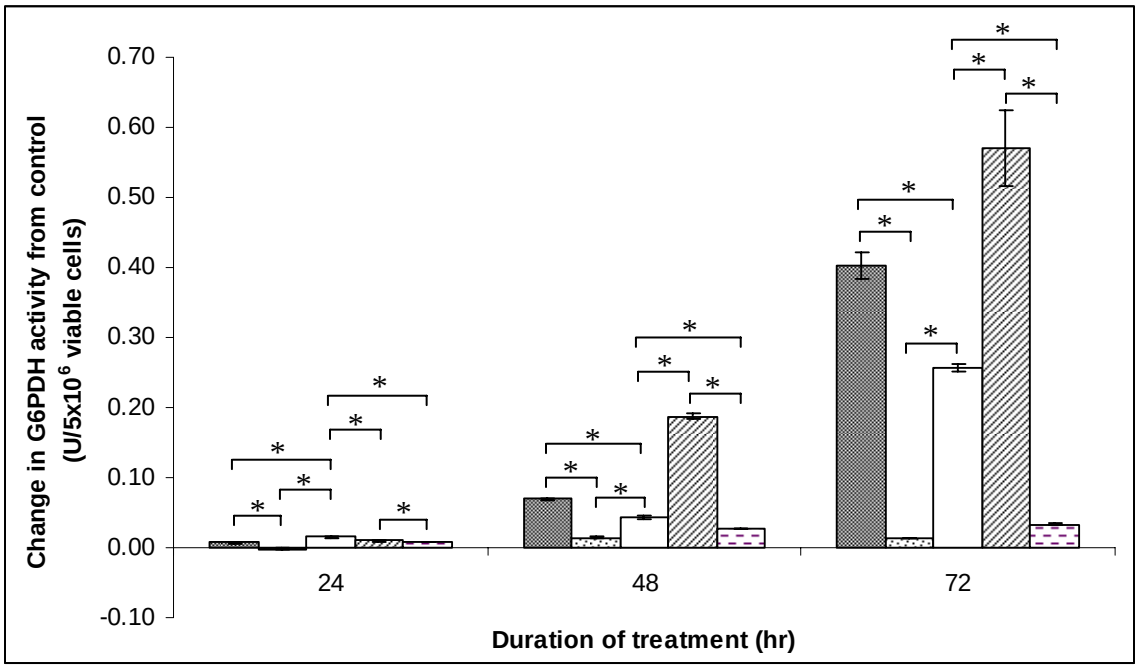

Figure 4.4: A comparison of OPP activities in Kato III cells following incubation with 5mM butyrate , 5mM propionate , 5mM SCFA combination , 10mM butyrate , and 10mM propionate . Data are expressed as 'change in G6PDH activity from control values' and presented as mean ± SEM (n = 9). *$p < 0.05$.

4.3.2: GSH availability

4.3.2.1: Butyrate decreased GSH availability in Kato III cells

Butyrate treatment significantly reduced the availability of GSH in Kato III cells after 24hr (1mM, 5mM, 10mM, $p < 0.001$, $n = 8$) and 48hr (1mM, 5mM, 10mM, $p < 0.001$, $n = 8$) compared to control values (Figure 4.5). However, after 72hr incubation, 1mM butyrate significantly increased GSH availability ($p < 0.05$, $n = 8$) while 10mM butyrate significantly reduced its availability ($p < 0.05$, $n = 8$).

4.3.2.2: Propionate reduced GSH availability in Kato III cells

Treatment of Kato III cells with propionate significantly decreased GSH availability after 24hr (1mM, 5mM 10mM, $p < 0.001$, $n = 8$) and 48hr (1mM, 5mM, 10mM, $p < 0.001$, $n = 8$) (Figure 4.6) compared to control values. GSH availability was not significantly different to control values after 72hr incubation with all concentrations of propionate ($p > 0.05$, $n = 8$). However, incubation with 10mM propionate resulted in a significantly lower GSH availability than 1mM propionate ($p < 0.01$, $n = 8$).

4.3.2.3: Butyrate/propionate combination treatment reduced GSH availability in Kato III cells

GSH availability was significantly decreased after 24hr ($p < 0.001$, $n = 8$), 48hr ($p < 0.001$, $n = 8$) and 72hr ($p < 0.01$, $n = 8$) compared to vehicle control cells (Figure 4.7).

4.3.2.4: Propionate decreased GSH availability greater than butyrate alone and SCFA combination treatment

After 24hr and 48hr incubation, GSH availability was decreased from control levels to the greatest extent following incubation with 5mM ($p < 0.05$) and 10mM ($p < 0.05$) propionate than 5mM and 10mM butyrate, respectively (Figure 4.8). Both butyrate (5mM, $p < 0.05$; 10mM $p <$

0.05) and propionate (5mM, $p < 0.05$; 10mM, $p < 0.05$) alone reduced GSH levels greater than 5mM butyrate/propionate mix after 24hr incubation. Greater reductions in GSH availability were observed after 48hr incubation with 5mM butyrate ($p < 0.05$), 5mM propionate ($p < 0.05$), and 10mM propionate compared to incubation with 5mM butyrate/propionate mix. However, after 72hr incubation, 10mM butyrate reduced GSH levels greater than 10mM propionate ($p < 0.05$) and 5mM butyrate/propionate combination ($p < 0.05$) treatment. Furthermore, SCFA combination treatment reduced GSH availability greater than 5mM butyrate ($p < 0.05$) and 5mM propionate ($p < 0.05$) alone (Figure 4.8).

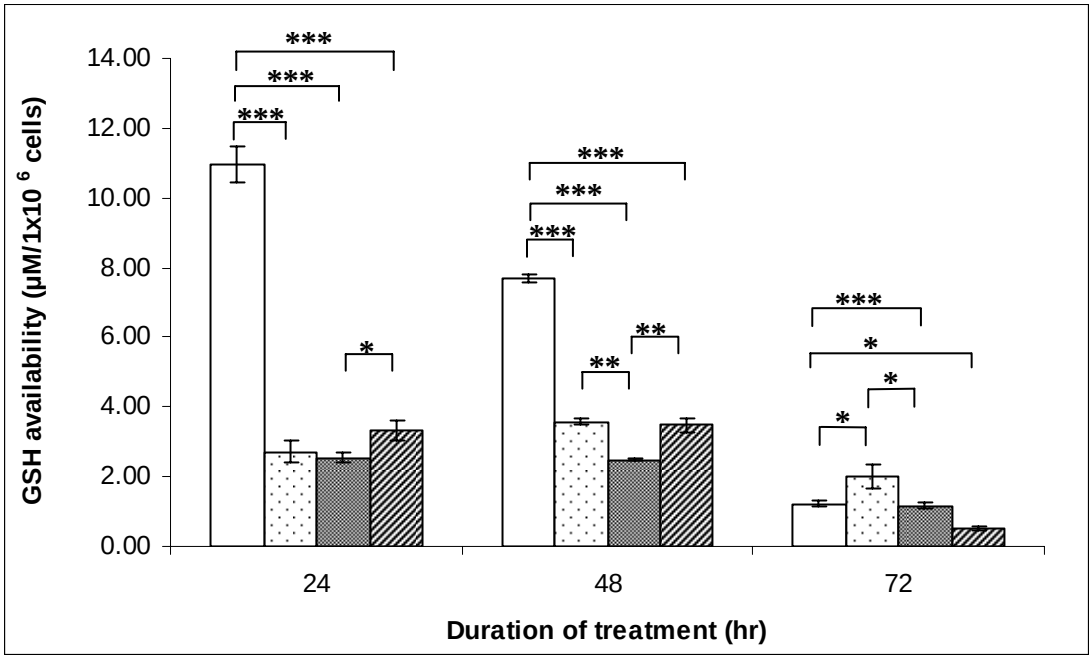

Figure 4.5: GSH availability in Kato III cells following incubation with 1mM ⬚, 5mM ▒ and 10mM ▨ butyrate compared to vehicle control □. Data are presented as mean ± SEM (n = 8). *p < 0.05, **p < 0.01, ***p < 0.001.

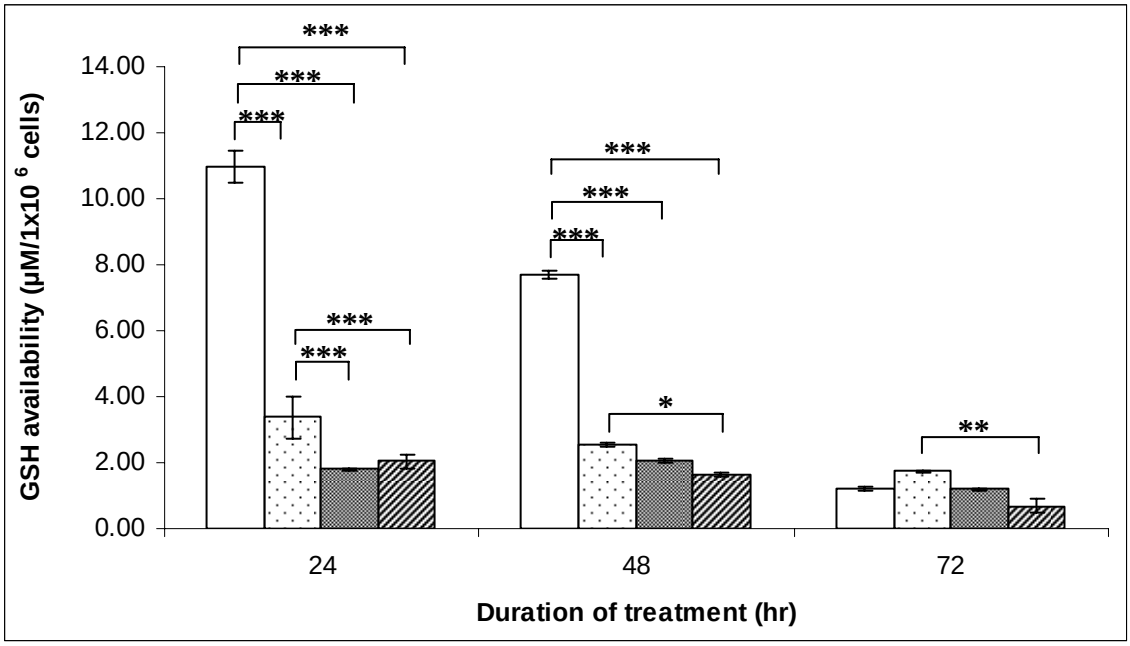

Figure 4.6: GSH availability in Kato III cells following incubation with 1mM ⬚, 5mM ▒ and 10mM ▨ propionate compared to vehicle control ⬚. Data are presented as mean ± SEM (n = 8). *p < 0.05, **p < 0.01, ***p < 0.001.

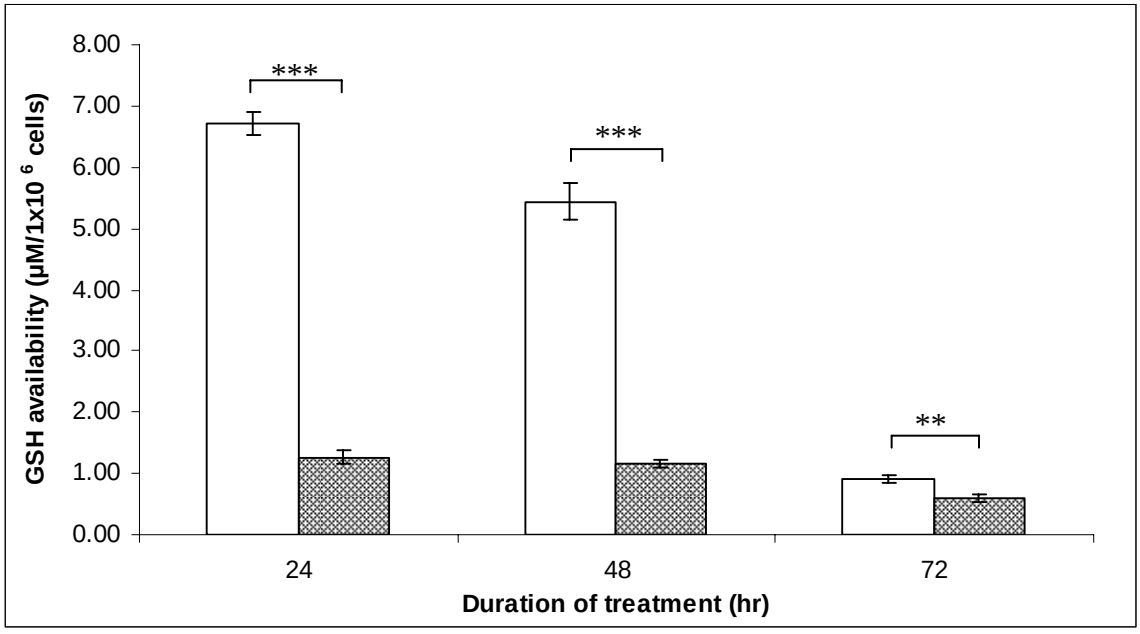

Figure 4.7: GSH availability in Kato III cells after incubation with 5mM butyrate/propionate combination ▩ or vehicle control □. Data are presented as mean ± SEM (n = 8). **p < 0.01, ***p < 0.001.

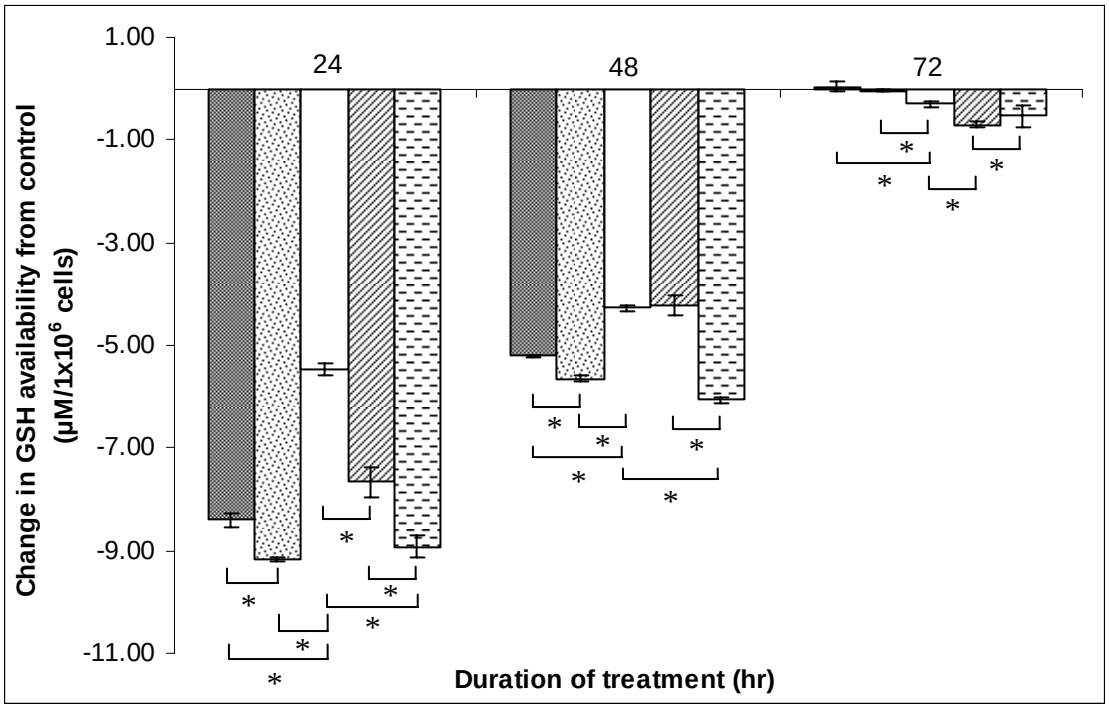

Figure 4.8: A comparison of GSH availability in Caco-2 cells following incubation with 5mM butyrate ▩, 5mM propionate ▨, 5mM SCFA combination □, 10mM butyrate ▨ and 10mM propionate ▨. Data are presented as mean ± SEM (n = 8). *$p < 0.05$.

4.3.4: 1-^{13}C-D-glucose oxidation

4.3.4.1: Butyrate altered 1-^{13}C-D-glucose oxidation in Kato III cells

1-^{13}C-D-glucose oxidation was significantly increased after 24hr ($p < 0.05$, $n = 7$) and 48hr ($p < 0.001$, $n = 4$) incubation with 5mM butyrate compared to control (24hr, $n = 4$; 48hr, $n = 4$) (Figure 4.9). However, after 72hr incubation with 5mM butyrate, 1-^{13}C-D-glucose oxidation was significantly decreased ($p < 0.001$) compared to vehicle control ($n = 5$).

4.3.4.2: Propionate increased 1-^{13}C-D-glucose oxidation in Kato III cells

Incubation with 5mM propionate significantly increased 1-^{13}C-D-glucose oxidation at all time points (24hr, $p < 0.001$, $n = 5$; 48hr, $p < 0.01$, $n = 5$; 72hr, $p < 0.01$, $n = 5$) compared to vehicle control ($n = 5$) (Figure 4.10).

4.3.4.3: SCFA combination treatment altered 1-^{13}C-D-glucose oxidation in Kato III cells

1-^{13}C-D-glucose oxidation was significantly increased after 24hr ($p < 0.001$) and 48hr (p 0.001) incubation with 5mM SCFA combination treatment compared to vehicle control ($n = 5$) (Figure 4.11). However, no differences were observed after 72hr incubation ($p > 0.05$, $n = 5$) compared to control ($n = 5$).

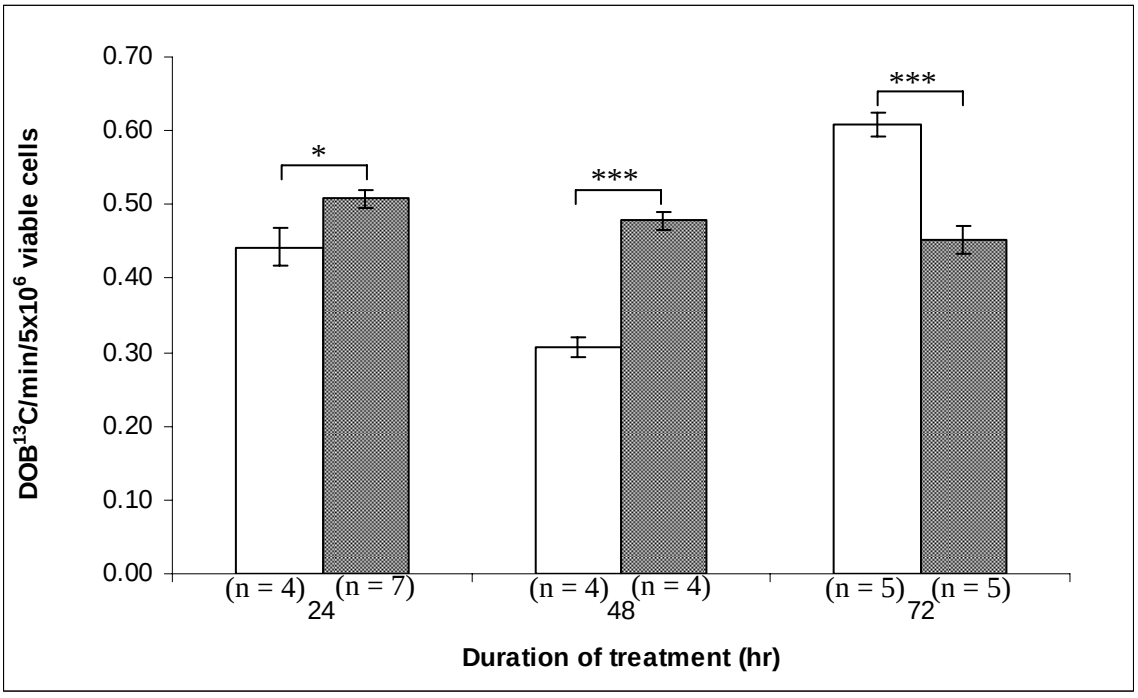

Figure 4.9: 1-^{13}C-D-glucose oxidation in Kato III cells following incubation with 5mM butyrate ▨ or vehicle control □. Data are presented as mean ± SEM (n = 4-7). *$p < 0.05$, ***$p < 0.001$.

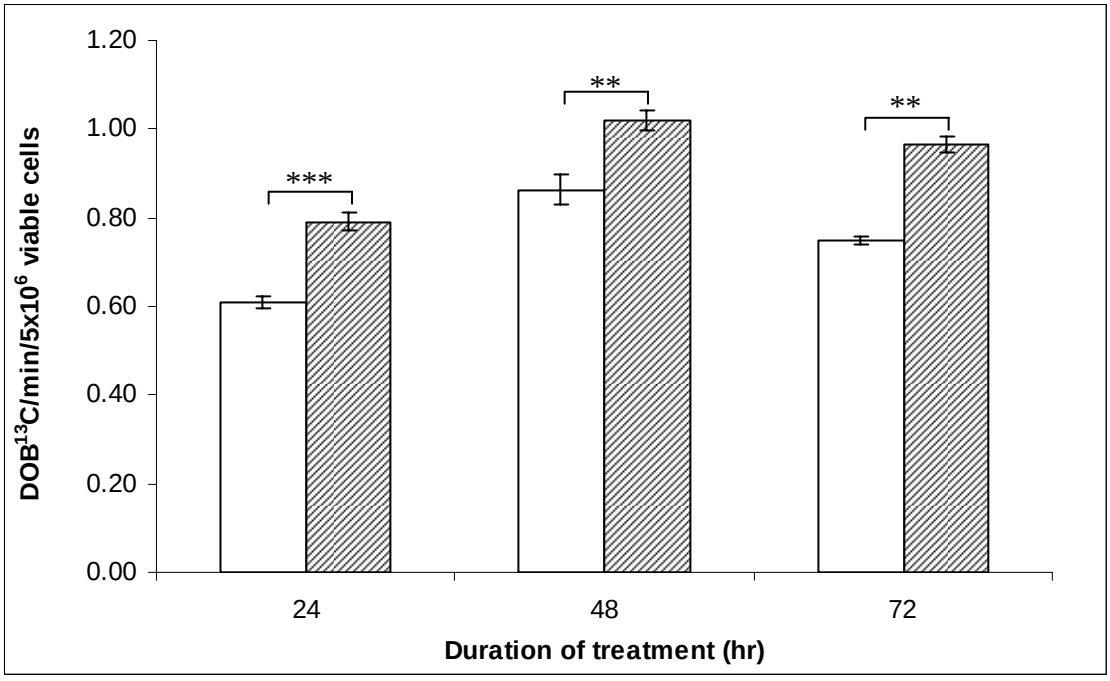

Figure 4.10: 1-^{13}C-D-glucose oxidation in Kato III cells following incubation with 5mM propionate ▨ and vehicle control □. Data are presented as mean ± SEM (n = 5). **p < 0.01, ***p < 0.001.

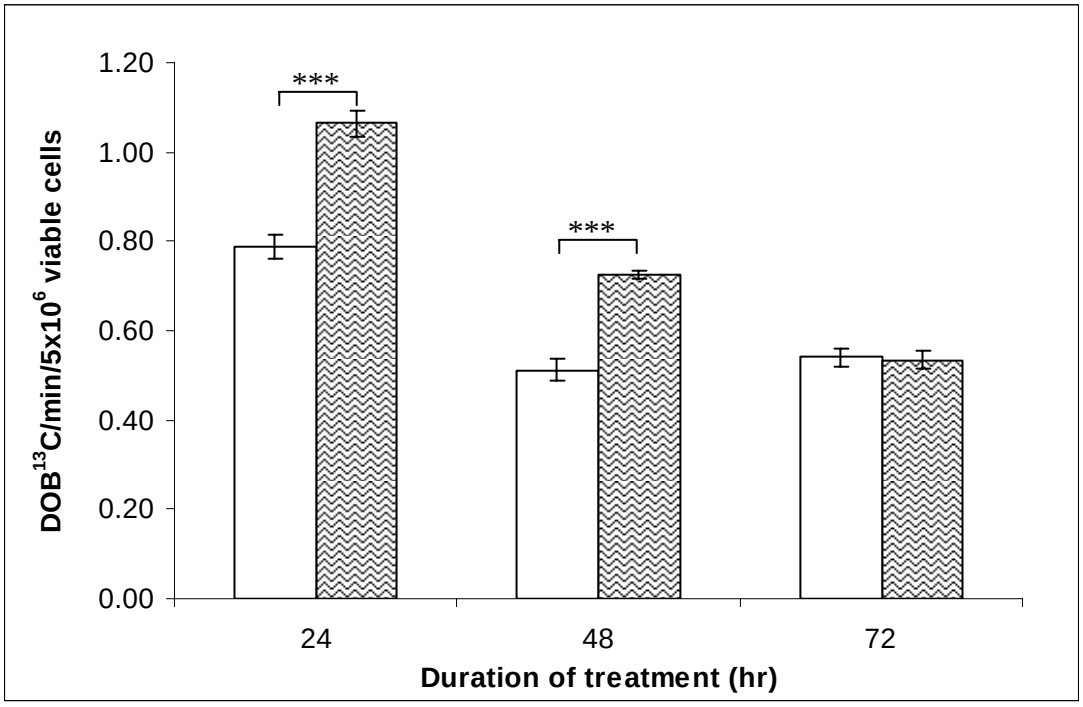

Figure 4.11: 1-^{13}C-D-glucose oxidation in Kato III cells following incubation with 5mM SCFA combination treatment ▨ or vehicle control □. Data are presented as mean ± SEM (n = 5). ***p < 0.001.

4.3.4.4: SCFA treatments differentially induced changes to 1-^{13}C-D-glucose oxidation in Kato III cells

After 24hr incubation, 5mM SCFA combination treatment increased 1-^{13}C-D-glucose oxidation to a significantly greater extent than 5mM propionate ($p < 0.01$) and 5mM butyrate ($p < 0.001$) alone (Figure 4.12). Additionally, incubation with 5mM propionate induced a greater increase in 1-^{13}C-D-glucose oxidation than 5mM butyrate ($p < 0.001$) after 24hr. No differences in 1-^{13}C-D-glucose oxidation were observed after 48hr incubation with any of the treatments ($p > 0.05$). However, 72hr incubation induced significant differences in 1-^{13}C-D-glucose oxidation in all treatment groups ($p < 0.001$) (Figure 4.12).

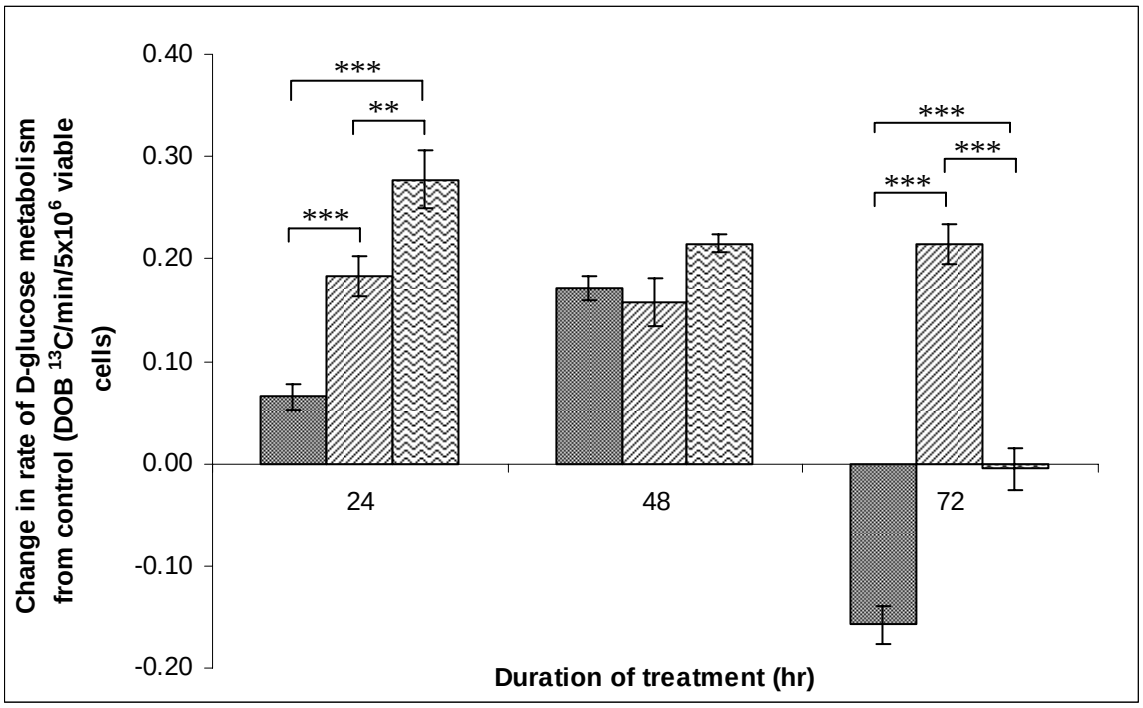

Figure 4.12: A comparison of the rates of D-glucose metabolism in Caco-2 cells following incubation with 5mM butyrate , 5mM propionate or 5mM SCFA combination treatments. Data are expressed as 'change in D-glucose metabolism from control levels' and presented as mean ± SEM (n = 5). **p < 0.01, ***p < 0.001.

4.4: Discussion

The current study assessed changes to intracellular protective mechanisms and D-glucose oxidation in the Kato III gastric cancer cell line by incubation with butyrate and propionate alone, or in combination. Butyrate, propionate and the combination of both SCFAs induced increases in OPP activity and decreases in GSH availability. D-glucose oxidation was initially increased by all SCFA treatments. However, differences in D-glucose oxidation were observed between SCFA treatments after longer term incubation.

Recent studies have suggested that butyrate may induce cell death by altering intracellular protective mechanisms, such as GSH availability [49-51]. In agreement with other investigators [49, 50], the current study observed significant reductions in GSH availability in Kato III cells immediately (24hr) following incubation with butyrate, propionate and the SCFA combination treatment. However, in contrast, the activity of the OPP was noted to increase, but not until after longer-term (48hr) incubation with all SCFA treatments.

The OPP is known to regulate intracellular redox state and provides NADPH for synthesis of GSH [21]. Therefore, an up-regulation in OPP activity would indicate an increase in the oxidative state of the intracellular environment, potentially due to an increase in ROS production. Up-regulation of G6PDH activity would also infer an increase in the production of GSH to offset the increased oxidant state of the cell. However, this study did not measure the rate of GSH synthesis and we can only propose that its production was increased. This will be undertaken in future studies. Therefore, it appeared that an up-regulation of OPP activity and, potentially, GSH synthesis was unable to prevent the marked depletion of GSH within these

cells. Hence, butyrate and propionate may have been able to induce cell death in this transformed cell line, in part, by decreasing their ability to protect themselves against oxidant attack.

The current study also investigated the ability of SCFAs to alter the rate of 1-^{13}C-D-glucose oxidation in Kato III gastric cancer cells. Boren *et al*. [38] previously reported that 72hr treatment of HT29 colon adenocarcinoma cells with butyrate led to decreases in [1,2-$^{13}C_2$]glucose oxidation. They suggested that this was due to the ability for HT29 cells to readily substitute the metabolism of glucose for butyrate, and this was not evident in the butyrate-resistant MIA (pancreatic adenocarcinoma) cell line. In agreement with Boren *et al*. [38], in the current study, 72hr treatment with butyrate decreased the rate of D-glucose oxidation in Kato III cells but this was not observed with propionate which increased D-glucose oxidation nor the SCFA combination treatment, which showed no effect on D-glucose oxidation. Interestingly, all SCFA treatments induced increases in the rate of D-glucose oxidation in Kato III cells by 24hr and 48hr incubation, with the combination SCFA treatment showing the greatest effect. Therefore, the current study has revealed that the early metabolic events ($\leq$ 48hr) during SCFA treatment in Kato III cells are characterised by increases in D-glucose oxidation and concomitant increases in OPP activity (by 48hr) that have not been previously reported. It appeared that by 72hr incubation, differences exist in the metabolic response of Kato III cells to treatment with butyrate or propionate, whereby butyrate decreased and propionate increased D-glucose oxidation in this cell type.

The results from the current study suggest that butyrate and propionate initially induce an increase in G6PDH activity, characterised by an increase in the oxidation of 1-^{13}C-D-glucose through the OPP, but this does not alter the rate of energy production by the tri-carboxylic acid (TCA) cycle. However, by 72hr incubation, butyrate is able to replace glucose for energy

production and thus cause the reduction in D-glucose oxidation apparent within this study. In contrast, the increased D-glucose oxidation rate by propionate appears mainly due to an increase in glucose utilisation by the OPP inferring that propionate is not substituted for glucose in energy production, at least at this time point. Moreover, the current study has shown that this effect is ameliorated when the SCFAs are combined, inferring that both SCFAs exert their individual effects on the metabolism of D-glucose and ultimately balances each other out. This study hypothesises that a mixed SCFA treatment would more closely mirror the luminal environment of the colon than either SCFA alone. A comparison between SCFA treatments in a colon cancer cell line will be discussed in a later chapter to investigate this hypothesis.

In conclusion, this study has documented the capacity for butyrate and propionate to reduce intracellular protective mechanisms and alter D-glucose metabolism in a Kato III gastric carcinoma cell line. It is proposed that butyrate and propionate have the potential to provide adjunctive therapies to current chemotherapeutic regimes for gastric cancer. Further studies are required to assess ROS production and GSH synthesis by butyrate and propionate in Kato III gastric cancer cells.

CHAPTER 5: Inhibition of intracellular protection by diethyl maleate and dehydroepiandrosterone enhances the sensitivity of Kato III cells to propionate but not butyrate-induced apoptosis

5.1: Introduction

Currently, an increase in the resistance of many cancers to chemotherapeutic agents is limiting our ability to effectively treat and eliminate these neoplasms [31, 95-97]. However, the mechanisms that induce chemoresistance are relatively unknown. Recent studies have proposed that the inhibition of cellular protective mechanisms, such as glutathione-S-transferase (GST), may increase the sensitivity of cancer cells to chemotherapeutic agents [31]. Hence, novel agents with the ability to increase the chemosensitivity of cancer cells are being investigated.

Recent work by Nakajima *et al*. [31] investigated the potential of a specific inhibitor of GST-π to alter the sensitivity of cholangiocarcinoma cells to anticancer drug therapy. It was observed that treatment with a GST-π-specific inhibitor led to a complete reversal of chemotherapeutic drug resistance in this cancer cell type. Other investigators have documented increases in oxidant-induced apoptosis both *in vivo* and *in vitro* using various drugs aimed at reducing intracellular protection and altering cellular redox state [50, 51, 93, 98, 99].

The SCFA butyrate has been shown to suppress GST-θ and GSH availability and reduce cell growth in the human colon cancer cell lines LT97 and HT29 [49, 51]. An up-regulation of the non-oxidative arm of the pentose phosphate pathway, by transaldolase over-expression, was shown by Banki *et al*. [93] to concomitantly reduce OPP activity and GSH availability in Jurkat human T cells. This, in turn, increased the sensitivity of this cell type to various apoptosis-inducing agents, including hydrogen peroxide and nitric oxide.

The current study aimed to investigate the effects of an inhibitor of cellular GSH, diethyl maleate (DEM), and a specific inhibitor of the OPP, dehydroepiandrosterone (DHEA), on SCFA-induced apoptosis in the Kato III gastric cancer cell line. It was hypothesised that DEM and DHEA would increase the sensitivity of Kato III cells to both butyrate and propionate by the reduction of cellular GSH availability. This study specifically assessed the induction of apoptosis and GSH availability in Kato III cells following treatment with DEM, DHEA, butyrate and/or propionate.

5.2: Materials and methods

5.2.1: Materials

Kato III gastric cancer cells were obtained from American Type Culture Collection (ATCC; Manassas, VA, USA). RPMI 1640, heat inactivated foetal bovine serum (FBS), Glutamax, Hepes (1M) and Penicillin/Streptomycin (10,000units/ml, 10,000µg/ml) were supplied by Invitrogen (Gibco®, Invitrogen Australia, VIC, Australia). Sodium butyrate, sodium propionate, potassium phosphate, $NADP^+$, EDTA disodium salt dihydrate, glucose-6-phosphate (G6P) disodium salt, 5,5'-dithiobis (2-nitrobenzoic acid; DTNB), G6PDH, DEM, DHEA and dimethyl sulfoxide (DMSO) were obtained from Sigma-Aldrich (Castle Hill, NSW, Australia). GSH and GSH reductase were supplied by Roche Diagnostics (Castle Hill, NSW, Australia). Annexin V FITC was obtained from Molecular Probes (Invitrogen Australia, VIC, Australia) and propidium iodide (PI) from Sigma-Aldrich. Stock (200mM) sodium butyrate and sodium propionate were prepared using sterile water and frozen at -20°C prior to the experiments. Stock DEM (1mM) and DHEA (1mM) were prepared by dilution in DMSO.

5.2.2: Kato III cell culture

Kato III cells were cultured in 20ml complete RPMI 1640 medium containing 10% FBS, 4mM Glutamax, 20mM Hepes and Penicillin/Streptomycin (73.5units/ml, 73.5ug/ml) in $75cm^2$ cell culture flasks. Cells were maintained at 37°C in a humidified 5% CO_2 incubator.

5.2.3: Experimental design

5.2.3.1: Initial experiments to determine the effects of DEM and DHEA on GSH availability in Kato III cells

In order to determine the effect of DEM and DHEA on GSH availability in Kato III cells, initial experiments involved treatment of Kato III cells with 0.1mM, 0.5mM and 1.0mM DEM or 0.05mM and 0.1mM DHEA. Kato III cells were seeded into 6-well plates ($3.5x10^6$ viable cells/well) in 5ml complete RPMI medium and incubated for 24hr at 37°C prior to treatment. Stock DEM or DHEA was then added to the wells in duplicate to give the appropriate final concentrations. DMSO (50µl) was added to vehicle control wells. Cells were incubated at 37°C and harvested using a cell scraper after 2hr, 24hr and 48hr. For the assessment of GSH availability, all cells from duplicate wells were removed and centrifuged at 156 x g for 5min. Supernatants were removed and cells resuspended in RPMI (1ml) and immediately assessed for viability and cell number by exclusion of 0.1% nigrosine before being frozen at -80°C until analysis. Results from this study were used to determine the concentration of DEM and DHEA and the timing of treatment to be utilised in the following experiment.

5.2.3.2: Assessment of apoptosis induction in Kato III cells after incubation with DEM, DHEA and/or SCFAs

Kato III cells were seeded into 6-well plates ($3.5x10^6$ viable cells/well) in 5ml complete RPMI medium and incubated for 24hr at 37°C prior to treatment. Stock DEM (10µl) or DHEA (100µl) were added to appropriate wells to provide final concentrations of 0.1mM. DMSO (50µl) was added to vehicle control wells. After 2hr incubation at 37°C, stock sodium butyrate (50µl) or sodium propionate (50µl) were added to appropriate wells in duplicate to give a final concentration of 5mM. Control wells received 50µl sterile water. Cells were incubated at 37°C

and harvested using a cell scraper after 24hr and 48hr. For assessments of apoptosis induction, aliquots of cell suspension (200µl) were immediately added to flow-assisted cell sorting (FACS) tubes and assessed as described below.

5.2.4: Assays

5.2.4.1: Morphological assessment of cell viability

In order to initially determine the concentrations of DEM and DHEA to be utilised in combination with SCFAs in later experiments, Kato III cell viability was firstly assessed morphologically by the exclusion of 0.1% nigrosine as described in Chapter 4 (4.2.4.1).

5.2.4.2: GSH availability

GSH availability was measured using the method described in Chapter 4 (4.2.4.3).

5.2.4.3: FACS assessment of cell viability

In order to assess the effect of combining DEM and DHEA with SCFA treatments on Kato III cells, cell viability was assessed using flow cytometry as described in Chapter 3 (3.2.4.1).

5.2.5: Statistics

All results are presented as mean ± SEM. Data were analysed at 24hr and 48hr time points using One-way ANOVA's with Student-Newman-Keuls *post-hoc* tests. Data were compared to appropriate control groups and within SCFA treatment groups only. No comparisons were made between SCFA groups as this was the focus of Chapter 3. All statistics were calculated using SigmaStat 3.0 (SYSTAT Software Inc., California, USA). Significance was assumed at $p < 0.05$.

5.3: Results

5.3.1: Initial validation experiments

5.3.1.1: Kato III viability was decreased after incubation with DEM

Interestingly, incubation with 0.1mM DEM significantly reduced Kato III cell viability after 2hr ($p < 0.05$, $n = 8$) but not at 0.5mM or 1.0mM ($p > 0.05$) compared to vehicle control values (Table 5.1). No difference in cell viability was observed after 24hr incubation with 0.1mM DEM ($p > 0.05$) although this concentration increased GSH availability after 48hr ($p < 0.05$). However, DEM treatment significantly decreased cell viability after 24hr (0.5mM, $p < 0.05$, $n = 8$; 1.0mM, $p < 0.05$, $n = 8$) and 48hr (0.5mM, $p < 0.05$, $n = 8$; 1.0mM, $p < 0.05$, $n = 8$) compared to control (Table 5.1).

5.3.1.2: Kato III viability was altered after incubation with DHEA

Incubation with DHEA significantly reduced cell viability after 2hr (0.1mM, $p < 0.05$, $n = 8$) and 24hr (0.05mM, $p < 0.05$, $n = 8$; 0.1mM, $p < 0.05$, $n = 8$) but not after 48hr (0.1mM, $p > 0.05$, $n = 8$) compared to control (Table 5.1). However, 48hr incubation with 0.05mM DHEA significantly increased cell viability ($p < 0.05$, $n = 8$). No other differences in cell viability were observed following DHEA treatment compared to control (Table 5.1).

Table 5.1: Morphological assessment of Kato III cell viability following incubation with DEM or DHEA.

Treatment	Concentration of treatment (mM)	Duration of treatment (hr) 2	48	72
Control	**0**	66.7 ± 3.2	65.5 ± 2.2	36.7 ± 1.6
DEM	**0.1**	58.3 ± 0.4	65.0 ± 3.6	48.1 ± 0.7[a]
DEM	**0.5**	67.3 ± 0.6	26.2 ± 0.7[ab]	1.5 ± 0.4[ab]
DEM	**1.0**	69.6 ± 0.8[b]	1.1 ± 0.1[abc]	0.0 ± 0.0[abc]
DHEA	**0.05**	55.6 ± 4.8[d]	50.6 ± 0.1[abcd]	40.7 ± 0.1[abcd]
DHEA	**0.1**	53.6 ± 0.1[abcd]	46.0 ± 0.5[abcde]	36.7 ± 1.5[bcde]

Data are presented as mean (%) ± SEM (n = 8). All treatment groups were compared to control ([a]$p < 0.05$), 0.1mM DEM ([b]$p < 0.05$), 0.5mM DEM ([c]$p < 0.05$), 1.0mM DEM ([d]$p < 0.05$) or 0.05mM DHEA ([e]$p < 0.05$).

5.3.1.3: DHEA reduced cell viability greater than an equivalent concentration of DEM

Incubation with 0.1mM DHEA reduced Kato III cell viability to a greater extent than 0.1mM DHEA after 2hr ($p < 0.05$), 24hr ($p < 0.05$) and 48hr ($p < 0.05$) (Table 5.1). With the exception of the 2hr incubation, all other concentrations of DEM (0.5mM and 1.0mM) reduced cell viability greater than 0.05mM and 0.1mM DHEA ($p < 0.05$).

5.3.2: GSH availability

5.3.2.1: GSH availability was significantly reduced in Kato III cells by DEM

Incubation of Kato III cells with DEM significantly reduced GSH availability after 2hr (0.1mM, $p < 0.001$, $n = 8$; 0.5mM, $p < 0.001$, $n = 8$; 1.0mM, $p < 0.001$, $n = 8$), 24hr (0.5mM, $p < 0.001$, $n = 8$; 1.0mM, $p < 0.001$, $n = 8$) and 48hr (0.1mM, $p < 0.001$, $n = 8$; 0.5mM, $p < 0.001$, $n = 8$; 1.0mM, $p < 0.001$, $n = 8$) compared to vehicle control values (Figure 5.1). After 2hr and 24hr incubation, 0.5mM and 1.0mM DEM reduced GSH availability in the Kato III cells to a greater extent than 0.1mM DEM ($p < 0.001$). This effect was not evident after 48hr incubation with DEM ($p > 0.05$).

5.3.2.2: GSH availability was significantly reduced in Kato III cells by DHEA

DHEA significantly decreased GSH availability in Kato III cells after 2hr (0.05mM, $p < 0.001$, $n = 8$; 0.1mM, $p < 0.001$, $n = 8$), 24hr (0.05mM, $p < 0.05$, $n = 8$) and 48hr (0.05mM, $p < 0.001$, $n = 8$; 0.1mM, $p < 0.001$, $n = 8$) compared to control (Figure 5.1). Incubation with 0.1mM DHEA for 2hr reduced GSH levels to a significantly greater extent than 0.05mM DHEA ($p < 0.001$). No other dose-dependent differences were observed.

5.3.2.3: Incubation with 0.1mM DEM or 0.1mM DHEA for 2hr reduced GSH availability to equivalent levels

From the results reported for changes to GSH availability (Figure 5.1) by DHEA and DEM it was evident that the greatest reductions in GSH availability followed 2hr treatment with both agents compared to control values. Additionally, no differences in GSH availability were observed after 2hr incubation with 0.1mM DEM and 0.05mM or 0.1mM DHEA ($p > 0.05$). Therefore, all proceeding experiments incubated Kato III cells with equivalent concentrations of DEM or DHEA (0.1mM) for 2hr prior to the addition of SCFAs.

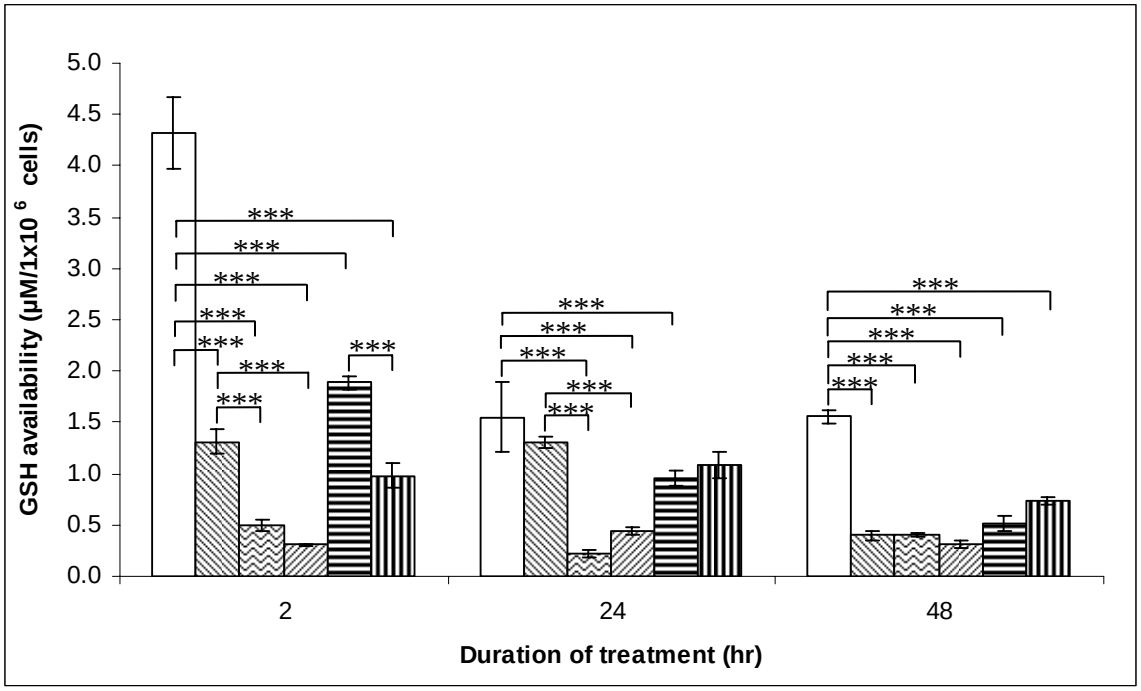

Figure 5.1: GSH availability in Kato III cells following incubation with 0.1mM DEM , 0.5mM DEM , 1.0mM DEM , 0.05mM DHEA or 0.1mM DHEA compared to vehicle control. Data are presented as mean ± SEM (n = 8). ***p < 0.001.

5.3.3: Cell viability: FACS analysis

5.3.3.1: Pre-incubation with DHEA, but not DEM, time-dependently reduced the viability of butyrate-treated Kato III cells

DEM (0.1mM, n = 6) and DHEA (0.1mM, n = 6) significantly reduced Kato III cell viability after 24hr incubation compared to vehicle control values ($p < 0.05$) (Table 5.2). DHEA treatment reduced cell viability to a greater extent than DEM treatment ($p < 0.05$). Butyrate treatment reduced cell viability after 24hr compared to control ($p < 0.05$, n = 6). No reduction in cell viability was noted in 24hr butyrate-treated Kato III cells that were pre-incubated with DEM compared to cells treated with butyrate alone ($p > 0.05$, n = 6). In contrast, pre-incubation of butyrate-treated Kato III cells with DHEA reduced Kato III cell viability to a significantly greater extent than butyrate alone after 24hr ($p < 0.05$, n = 6). However, 24hr DHEA and butyrate treated cells showed higher cell viabilities than cells treated with DHEA alone ($p < 0.05$) (Table 5.2).

Treatment of Kato III cells with butyrate alone for 48hr significantly decreased cell viability compared to control cells ($p < 0.05$, n = 6) (Table 5.2). No difference in cell viability was observed in butyrate-treated cells pre-incubated with either DEM or DHEA and Kato III cells treated with butyrate alone ($p > 0.05$, n = 6). However, butyrate-treated cells that were pre-incubated with DEM or DHEA had significantly lower cell viability than cells treated with DEM or DHEA alone, respectively ($p < 0.05$).

Table 5.2: Percentage Kato III cell viability following incubation with DEM, DHEA and/or SCFAs.

Pre-incubation treatment	SCFA treatment	Concentration of SCFA treatment (mM)	Duration of treatment (hr)	
			24	48
Control	**Control**	**0**	71.7 ± 2.4	64.8 ± 0.7
0.1mM DEM	**Control**	**0**	52.4 ± 2.7[a]	57.0 ± 2.0[a]
0.1mM DHEA	**Control**	**0**	30.4 ± 0.5[ab]	27.5 ± 0.9[ab]
Control	**Butyrate**	**5**	57.0 ± 0.8[ac]	21.3 ± 2.0[abc]
0.1mM DEM	**Butyrate**	**5**	53.3 ± 0.8[ac]	16.1 ± 1.9[abc]
0.1mM DHEA	**Butyrate**	**5**	35.5 ± 0.8[abcde]	22.0 ± 2.4[abc]
Control	**Propionate**	**5**	54.8 ± 1.0[abc]	59.0 ± 0.5[ac]
0.1mM DEM	**Propionate**	**5**	60.6 ± 0.6[acf]	46.9 ± 1.0[abcf]
0.1mM DHEA	**Propionate**	**5**	35.8 ± 0.8[abcfg]	38.3 ± 2.6[abcfg]

Data are presented as mean (%) ± SEM (n = 6). Treatment groups were compared to the appropriate control group and within each SCFA group only. Statistical significance between groups is presented as follows: [a]$p < 0.05$ (vs. control pre-incubation + control SCFA), [b]$p < 0.05$ (vs. 0.1mM DEM + control SCFA), [c]$p < 0.05$ (vs. 0.1mM DHEA + control SCFA, [d]$p < 0.05$ (vs. control pre-incubation + 5mM butyrate), [e]$p < 0.05$ (vs. 0.1mM DEM + 5mM butyrate, [g]$p < 0.05$ (vs. control pre-incubation + 5mM propionate), and [h]$p < 0.05$ (vs. 0.1mM DEM + 5mM propionate).

5.3.3.2: Pre-incubation with DEM and DHEA time-dependently reduced the viability of propionate-treated Kato III cells

Kato III cell viability was significantly reduced after 24hr incubation with propionate ($p < 0.05$, n= 6) (Table 5.2). Pre-incubation of 24hr propionate-treated Kato III cells with DEM led to a significant increase in cell viability compared to cells treated with butyrate alone ($p < 0.05$, $n = 6$). In contrast, the pre-incubation of 24hr propionate-treated Kato III cells with DHEA reduced cell viability to a significantly greater extent than propionate treatment alone ($p < 0.05$, $n = 6$). However, DEM and DHEA pre-treatment of 24hr propionate-treated cells induced significantly higher cell viabilities than either DEM or DHEA alone, respectively ($p < 0.05$).

Incubation with propionate alone for 48hr significantly reduced cell viability compared to vehicle control values ($p < 0.05$, $n = 6$) (Table 5.2). DHEA treatment (48hr) reduced cell viability to a greater extent than DEM treatment ($p < 0.05$). The pre-incubation of 48hr propionate-treated cells with DEM and DHEA reduced Kato III viability compared to propionate treatment alone ($p < 0.05$, $n = 6$). In addition, pre-incubation of propionate-treated Kato III cells with DEM reduced cell viability to a significantly greater extent than DEM treatment alone ($p < 0.05$). In contrast, it was observed that pre-incubation of propionate-treated cells with DHEA did not reduce cell viability to the extent of DHEA treatment alone ($p < 0.05$) (Table 5.2).

5.3.3.3: Pre-incubation with DHEA time-dependently increased total apoptosis (TA) in butyrate treated Kato III cells

Incubation with DEM, DHEA or butyrate significantly increased the proportion of TA in Kato III cells compared to vehicle control values after 24hr ($p < 0.05$) (Table 5.3). Pre-incubation of butyrate-treated cells with DEM had no effect on TA compared to cells treated with propionate alone ($p > 0.05$) and DEM alone ($p < 0.05$). In contrast, pre-incubation of 24hr butyrate-treated

cells with DHEA increased TA to a significantly greater extent than butyrate treatment alone ($p < 0.05$). However, no difference in the percentage of TA was observed between cells treated with DHEA alone or in combination with butyrate ($p < 0.05$) (Table 5.3).

Incubation with DHEA and butyrate alone for 24hr, but not DEM, significantly increased the proportion of TA in Kato III cells compared to control values ($p < 0.05$) (Table 5.3). No differences in TA were observed after pre-incubation of 48hr butyrate-treated cells with DEM or DHEA compared to cells treated with butyrate alone ($p > 0.05$). However, pre-incubation of butyrate-treated cells with DEM and DHEA induced a greater proportion of TA in Kato III cells than DEM or DHEA alone ($p < 0.05$).

Table 5.3: Percentage total apoptosis in Kato III cells following incubation with DEM, DHEA and/or SCFAs.

Pre-incubation treatment	SCFA treatment	Concentration of SCFA treatment (mM)	Duration of treatment (hr) 24	48
Control	**Control**	**0**	13.0 ± 0.9	20.0 ± 1.9
0.1mM DEM	**Control**	**0**	27.9 ± 1.5[a]	20.3 ± 1.6
0.1mM DHEA	**Control**	**0**	40.8 ± 2.0[ab]	35.1 ± 3.9[ab]
Control	**Butyrate**	**5**	27.8 ± 1.2[ac]	48.2 ± 3.3[abc]
0.1mM DEM	**Butyrate**	**5**	27.2 ± 1.1[ac]	52.0 ± 3.0[abc]
0.1mM DHEA	**Butyrate**	**5**	37.3 ± 2.1[abde]	46.4 ± 4.1[abc]
Control	**Propionate**	**5**	31.2 ± 1.9[ac]	25.5 ± 1.3[c]
0.1mM DEM	**Propionate**	**5**	26.8 ± 0.8[ac]	32.2 ± 1.5[ab]
0.1mM DHEA	**Propionate**	**5**	38.5 ± 2.1[abfg]	37.2 ± 3.1[abf]

Data are presented as mean (%) ± SEM (n = 6). Treatment groups were compared to the appropriate control group and within each SCFA group only. Statistical significance between groups is presented as follows: [a]$p < 0.05$ (vs. control pre-incubation + control SCFA), [b]$p < 0.05$ (vs. 0.1mM DEM + control SCFA), [c]$p < 0.05$ (vs. 0.1mM DHEA + control SCFA, [d]$p < 0.05$ (vs. control pre-incubation + 5mM butyrate), [e]$p < 0.05$ (vs. 0.1mM DEM + 5mM butyrate, [g]$p < 0.05$ (vs. control pre-incubation + 5mM propionate), and [h]$p < 0.05$ (vs. 0.1mM DEM + 5mM propionate).

5.3.3.4: Pre-incubation with DHEA increased TA in propionate-treated Kato III cells

Incubation of Kato III cells with propionate for 24hr significantly increased the proportion of TA compared to vehicle control values ($p < 0.05$) (Table 5.3). No differences in TA were observed in propionate-treated cells pre-incubated with DEM compared to cells treated with propionate or DEM alone ($p > 0.05$). However, the pre-incubation of 24hr propionate-treated cells with DHEA led to a significantly increased proportion of TA in Kato III cells than propionate treatment alone ($p < 0.05$) but this did not differ significantly to cells treated with DHEA alone ($p > 0.05$) (Table 5.3).

The incubation of Kato III cells with propionate for 48hr did not change the proportion of cells undergoing TA compared to vehicle control values ($p > 0.05$) (Table 5.3). No difference in the percentage of TA was observed in propionate-treated cells pre-incubated with DEM and cells treated with propionate alone ($p > 0.05$). However, TA was significantly increased in propionate-treated Kato III cells pre-incubated with DEM compared to cells treated with DEM alone ($p < 0.05$). In contrast, pre-incubation of 48hr propionate-treated cells with DHEA led to significantly greater TA than cells treated with propionate alone ($p < 0.05$) but not DHEA alone ($p > 0.05$).

5.3.3.5: Pre-incubation with DHEA and DEM dose- and time-dependently altered the proportion of late apoptosis (LA) in butyrate-treated Kato III cells

The incubation of Kato III cell for 24hr with DEM and DHEA significantly reduced the proportion of LA compared to vehicle control values ($p < 0.05$) (Table 5.4). DHEA treatment increased LA to a significantly greater extent than DEM treatment ($p < 0.05$).

Table 5.4: Percentage of Kato III cells undergoing late apoptosis following incubation with DEM, DHEA and/or SCFAs.

Pre-incubation treatment	SCFA treatment	Concentration of SCFA treatment (mM)	Duration of treatment (hr)	
			24	48
Control	**Control**	**0**	8.4 ± 0.7	9.2 ± 1.1
0.1mM DEM	**Control**	**0**	19.2 ± 0.9[a]	13.4 ± 1.2
0.1mM DHEA	**Control**	**0**	34.2 ± 1.4[ab]	30.2 ± 3.1[ab]
Control	**Butyrate**	**5**	11.0 ± 0.5[bc]	27.0 ± 1.5[ab]
0.1mM DEM	**Butyrate**	**5**	16.6 ± 0.7[acd]	21.3 ± 1.2[abc]
0.1mM DHEA	**Butyrate**	**5**	30.7 ± 1.6[abcde]	39.4 ± 3.4[abcde]
Control	**Propionate**	**5**	18.5 ± 1.1[ac]	13.1 ± 0.9[c]
0.1mM DEM	**Propionate**	**5**	16.6 ± 0.4[ac]	16.5 ± 0.8[ac]
0.1mM DHEA	**Propionate**	**5**	31.4 ± 1.4[abfg]	26.3 ± 1.9[abfg]

Data are presented as mean (%) ± SEM (n = 6). Treatment groups were compared to the appropriate control group and within each SCFA group only. Statistical significance between groups is presented as follows: [a]$p < 0.05$ (vs. control pre-incubation + control SCFA), [b]$p < 0.05$ (vs. 0.1mM DEM + control SCFA), [c]$p < 0.05$ (vs. 0.1mM DHEA + control SCFA, [d]$p < 0.05$ (vs. control pre-incubation + 5mM butyrate), [e]$p < 0.05$ (vs. 0.1mM DEM + 5mM butyrate, [g]$p < 0.05$ (vs. control pre-incubation + 5mM propionate), and [h]$p < 0.05$ (vs. 0.1mM DEM + 5mM propionate).

Incubation with butyrate did not significantly alter the percentage of LA in Kato III cells compared to control ($p > 0.05$) (Table 5.4). Pre-incubation of 24hr butyrate-treated cells with DEM significantly increased LA compared to cells treated with propionate alone ($p < 0.05$) but not DEM alone ($p > 0.05$). It was also observed that pre-incubation of 24hr butyrate-treated cells with DHEA led to a significantly greater percentage of LA than cells treated with butyrate alone ($p < 0.05$) but less than those treated with DHEA alone ($p < 0.05$).

5.3.3.6: Pre-incubation with DEM or DHEA differentially and time-dependently altered the proportion of early apoptosis (EA) in butyrate-treated Kato III cells

Treatment of Kato III cells with DEM, DHEA or butyrate increased the percentage of Kato III cells in EA after 24hr (0.1mM DEM, $p < 0.05$; 0.1mM DHEA, $p < 0.05$; 5mM butyrate, $p < 0.05$) compared to vehicle control values (Table 5.5). No differences in the percentage of Kato III cells in EA was noted between cells treated with DEM or DHEA ($p > 0.05$). Pre-incubation of butyrate-treated Kato III cells with DEM or DHEA significantly reduced the percentage of EA compared to cells treated with butyrate alone ($p < 0.05$) but not when compared to DEM or DHEA alone ($p > 0.05$).

After 48hr incubation, treatment with DEM had no significant effect on the percentage of EA in Kato III cells compared to vehicle control ($p > 0.05$) (Table 5.5). However, treatment of Kato III cells with DHEA significantly decreased the proportion of cells undergoing EA compared to vehicle control ($p < 0.05$). No significant difference was observed in the proportion of EA caused by DEM or DHEA treatment ($p > 0.05$).

Table 5.5: Percentage of Kato III cells undergoing early apoptosis following incubation with DEM, DHEA and/or SCFAs.

Pre-incubation treatment	SCFA treatment	Concentration of SCFA treatment (mM)	Duration of treatment (hr)	
			24	48
Control	**Control**	**0**	4.5 ± 0.6	10.7 ± 0.8
0.1mM DEM	**Control**	**0**	8.7 ± 0.9[a]	6.9 ± 0.5
0.1mM DHEA	**Control**	**0**	6.6 ± 0.7[a]	4.9 ± 0.8[a]
Control	**Butyrate**	**5**	16.7 ± 0.8[abc]	21.3 ± 2.0[abc]
0.1mM DEM	**Butyrate**	**5**	10.6 ± 0.6[acd]	30.7 ± 1.9[abcd]
0.1mM DHEA	**Butyrate**	**5**	6.7 ± 0.7[de]	7.0 ± 0.7[de]
Control	**Propionate**	**5**	12.7 ± 0.9[abc]	12.4 ± 0.5[bc]
0.1mM DEM	**Propionate**	**5**	10.2 ± 0.4[acf]	15.6 ± 0.8[abcf]
0.1mM DHEA	**Propionate**	**5**	7.1 ± 0.8[fg]	10.9 ± 1.4[bcg]

Data are presented as mean (%) ± SEM (n = 6). Treatment groups were compared to the appropriate control group and within each SCFA group only. Statistical significance between groups are presented as follows: [a]$p < 0.05$ (vs. control pre-incubation + control SCFA), [b]$p < 0.05$ (vs. 0.1mM DEM + control SCFA), [c]$p < 0.05$ (vs. 0.1mM DHEA + control SCFA, [d]$p < 0.05$ (vs. control pre-incubation + 5mM butyrate), [e]$p < 0.05$ (vs. 0.1mM DEM + 5mM butyrate, [g]$p < 0.05$ (vs. control pre-incubation + 5mM propionate), and [h]$p < 0.05$ (vs. 0.1mM DEM + 5mM propionate).

Butyrate treatment significantly increased the proportion of EA in Kato III cells compared to control values ($p < 0.05$) (Table 5.5). Pre-incubation of butyrate-treated cells with DEM significantly increased the percentage of EA in Kato III cells compared to butyrate treatment alone ($p < 0.05$) and DEM treatment alone ($p < 0.05$). In contrast, pre-incubation of 48hr butyrate-treated cells with DHEA significantly reduced the proportion of EA in Kato III cells compared to butyrate treatment alone ($p < 0.05$) but not DHEA treatment alone ($p > 0.05$).

5.3.3.7: Pre-incubation with DEM or DHEA differentially and time-dependently altered the percentage EA in propionate-treated Kato III cells

Incubation with propionate significantly increased the percentage of EA in Kato III cells after 24hr incubation compared to vehicle control values ($p < 0.05$) (Table 5.5). The pre-incubation of propionate-treated Kato III cells with DEM or DHEA significantly reduced the percentage of EA compared to propionate treatment alone ($p < 0.05$) but this was not different to DEM or DHEA alone, respectively ($p > 0.05$).

Incubation of Kato III cells with propionate for 48hr had no significant effect on EA compared to control values ($p > 0.05$) (Table 5.5). It was observed that pre-incubation of 48hr propionate-treated cells with DEM led to a significant increase in EA compared to propionate treatment alone ($p < 0.05$) and DEM treatment alone ($p < 0.05$). Pre-incubation of 48hr propionate-treated Kato III cells with DHEA increased the proportion of EA in Kato III cells compared to DHEA treatment alone ($p < 0.05$) but not propionate treatment alone ($p > 0.05$).

5.3.3.8: Pre-incubation with DHEA, but not DEM, time-dependently increased the percentage of necrosis in butyrate-treated Kato III cells

The incubation of Kato III cells with DHEA for 24hr significantly increased the percentage of cells undergoing necrosis compared to vehicle control values ($p < 0.05$) and compared to treatment with DEM ($p < 0.05$) (Table 5.6). No differences were observed after 24hr treatment with DEM or butyrate compared to control ($p > 0.05$). Pre-incubation of 24hr butyrate-treated cells with DEM had no significant effect on the induction of necrosis in Kato III cells compared to butyrate treatment alone ($p > 0.05$) and DEM treatment alone ($p > 0.05$). However, pre-incubation of 24hr butyrate-treated cells with DHEA significantly increased the proportion of necrotic Kato III cells compared to butyrate treatment alone ($p < 0.05$) but not DHEA treatment alone ($p > 0.05$) (Table 5.6).

Incubation of Kato III cells with DEM, DHEA or butyrate for 48hr significantly increased the percentage of necrosis compared to control values ($p < 0.05$) (Table 5.6). Treatment with DHEA induced necrosis to a significantly greater extent than DEM treatment ($p < 0.05$). Pre-incubation of 48hr butyrate-treated Kato III cells with DEM had no significant effect on the proportion of necrotic cells compared to butyrate treatment alone ($p > 0.05$) but this was significantly higher than DEM treatment alone ($p < 0.05$). Furthermore, the pre-incubation of 48hr butyrate-treated cells with DHEA had no significant effect on necrosis in Kato III cells compared to butyrate treatment alone ($p > 0.05$) and DHEA treatment alone ($p > 0.05$) (Table 5.6).

Table 5.6: Percentage of Kato III cells undergoing necrosis following incubation with DEM, DHEA and/or SCFAs.

Pre-incubation treatment	SCFA treatment	Concentration of SCFA treatment (mM)	Duration of treatment (hr) 24	48
Control	**Control**	**0**	15.4 ± 2.4	15.3 ± 1.4
0.1mM DEM	**Control**	**0**	19.7 ± 2.8	22.7 ± 1.0[a]
0.1mM DHEA	**Control**	**0**	28.8 ± 1.8[ab]	37.4 ± 3.1[ab]
Control	**Butyrate**	**5**	15.2 ± 1.5[c]	32.1 ± 1.8[ab]
0.1mM DEM	**Butyrate**	**5**	19.5 ± 1.4[c]	31.9 ± 1.2[ab]
0.1mM DHEA	**Butyrate**	**5**	27.2 ± 1.9[abd]	31.6 ± 2.4[ab]
Control	**Propionate**	**5**	14.0 ± 1.2[c]	15.5 ± 1.1[bc]
0.1mM DEM	**Propionate**	**5**	12.6 ± 0.6[c]	20.9 ± 1.2[acf]
0.1mM DHEA	**Propionate**	**5**	25.7 ± 2.7[afg]	24.4 ± 2.0[acf]

Data are presented as mean (%) ± SEM (n = 6). Treatment groups were compared to the appropriate control group and within each SCFA group only. Statistical significance between groups is presented as follows: [a]$p < 0.05$ (vs. control pre-incubation + control SCFA), [b]$p < 0.05$ (vs. 0.1mM DEM + control SCFA), [c]$p < 0.05$ (vs. 0.1mM DHEA + control SCFA, [d]$p < 0.05$ (vs. control pre-incubation + 5mM butyrate), [e]$p < 0.05$ (vs. 0.1mM DEM + 5mM butyrate, [g]$p < 0.05$ (vs. control pre-incubation + 5mM propionate), and [h]$p < 0.05$ (vs. 0.1mM DEM + 5mM propionate).

5.3.3.9: Pre-incubation with DEM and DHEA time-dependently increased necrosis in propionate-treated Kato III cells

Propionate treatment for 24hr had no significant effect on necrosis in Kato III cells compared to vehicle control cells ($p > 0.05$) (Table 5.6). Pre-incubation of 24hr propionate-treated cells with DEM had no effect on the percentage of necrotic Kato III cells compared to propionate treatment alone ($p > 0.05$) but induced significantly less necrosis than DEM treatment alone ($p < 0.05$). In contrast, pre-incubation of propionate-treated cells with DHEA significantly increased the percentage of necrosis in Kato III cells compared to cells treated with propionate alone ($p < 0.05$) but not DHEA treatment alone ($p > 0.5$).

No significant change in the percentage of necrotic Kato III cells was observed after 48hr treatment with propionate compared to vehicle control ($p > 0.05$) (Table 5.6). Pre-incubation of 48hr propionate-treated cells with DEM significantly increased the proportion of Kato III cells undergoing necrosis compared to propionate treatment alone ($p < 0.05$) but not DEM treatment alone ($p > 0.05$). In addition, pre-incubation of 48hr propionate-treated cells with DHEA significantly increased necrosis in Kato III cells compared to propionate treatment alone ($p < 0.05$) but this was significantly less than DHEA treatment alone (Table 5.6).

5.4: Discussion

The current study investigated the potential for two inhibitors of cellular protection to enhance the sensitivity of Kato III gastric cancer cells to butyrate and propionate-induced cell death. It was observed that pre-incubation of Kato III cells with DEM or DHEA differentially and time-dependently altered the effects of treatment with butyrate and propionate. However, this study also noted that DHEA treatment alone reduced cell viability greater than when it was combined with either butyrate or propionate.

The OPP and intracellular GSH availability are considered extremely important factors controlling cellular redox status and intracellular protection from ROS [21-23, 91]. Therefore, the current study hypothesised that the inhibition of intracellular protective mechanisms by attenuation of OPP activity and GSH availability would enhance the sensitivity of Kato III cells to SCFA-induced cell death. Thus, Kato III cells were pre-incubated with DEM, a glutathione-depleting agent [96, 100], or DHEA, a specific inhibitor of G6PDH activity [22], followed by treatment with butyrate or propionate.

Initial experiments revealed significant reductions in GSH availability and cell viability (assessed morphologically) following treatment with either DEM or DHEA. Indeed, 24hr and 48hr incubation of Kato III cells with 1.0mM DEM reduced cell viability to 1.1% and 0%, respectively. It was determined that equivalent concentrations of DEM and DHEA (0.1mM) caused similar reductions in GSH availability after 2hr incubation. Therefore, a 2hr incubation of Kato III cells with either 0.1mM DEM or 0.1mM DHEA was utilised in the subsequent experiments using SCFAs.

The present study demonstrated that pre-incubation of Kato III cells with DHEA, but not DEM, significantly enhanced the ability of butyrate to reduce cell viability after 24hr incubation. However, this was not significantly greater than the reductions in cell viability gained with DHEA treatment alone at this time point. Therefore, after 24hr incubation, this study has revealed that treatment with DHEA alone reduced cell viability to a greater extent than concomitant treatment with DHEA and butyrate. Interestingly, after 48hr, butyrate-treated cells that were pre-incubated with DEM or DHEA showed significantly less viability than cells treated with DEM or DHEA alone. However, this was not significantly different to the viability of cells treated with butyrate alone. It is suggested that 48hr time point represents an overall indication of treatment effect within the current study. Hence, the results of the current study suggest that the greatest reduction in cell viability was achieved by butyrate treatment alone.

When propionate treated Kato III cells were pre-incubated with DEM or DHEA, differential and time-dependent changes to cell viability were observed compared to propionate treatment alone. After 24hr treatment, pre-incubation of propionate-treated Kato III cells with DEM or DHEA led to a significant increase and decrease in cell viability, respectively. However, the viability of Kato III cells following treatment with DHEA and propionate was significantly higher than following DHEA treatment alone at this time point. Incubation for 48hr resulted in significant decreases in cell viability when propionate-treated Kato III cells were pre-incubated with DEM or DHEA. Again, however, cell viabilities were significantly higher in these treatment groups than DHEA treatment alone. Therefore, the greatest reduction in cell viability following propionate treatment was obtained when Kato III cells were pre-incubated with DHEA. Moreover, DHEA treatment alone reduced cell viability to a greater extent than in combination with propionate treatment.

In order to determine the cellular processes that ultimately culminated in the reductions in Kato III cell viability within this study, cell death mechanisms were classified into four categories by FACS analysis (TA, LA, EA and necrosis). The following discussion will refer to results from the 48hr time point only, as discussed.

The current study observed that the percentage of TA in Kato III cells was not significantly affected by pre-incubation of butyrate-treated cells with DEM or DHEA. However, pre-incubation of Kato III cells with DEM led to a significant increase in EA. In contrast, pre-incubation of butyrate-treated cells with DHEA induced a significant reduction in LA and a significant increase in EA. No significant change to the percentage of necrotic Kato III cells was observed when butyrate-treated cells were pre-incubated with DEM or DHEA. Therefore, these results suggested that although DEM and DHEA induced differential mechanisms of cell death, they did not significantly enhance the sensitivity of Kato III cells to butyrate treatment.

The present study also revealed that pre-incubation of propionate-treated Kato III cells with DHEA, but not DEM, led to a significant increase in TA. Further assessment of apoptotic events by FACS analysis indicated that pre-incubation with DHEA led to a significant increase in LA but not EA. Pre-incubation of propionate-treated cells with DEM increased the percentage of EA, however, this did not increase the overall proportion of apoptosis (TA) within this study. Additionally, the percentage of necrotic Kato III cells was significantly increased by pre-incubation of propionate-treated cells with DEM or DHEA. Therefore, pre-incubation with DEM enhanced the sensitivity of Kato III cells to propionate by primarily increasing the amount of necrosis-associated cell death. DHEA pre-treatment enhanced the sensitivity of Kato III cells to propionate by concomitantly increasing the percentage of LA and necrosis in this cell type.

Previous studies have proposed that pharmacologically induced changes to cellular GSH availability could alter a cell's sensitivity to ROS during various disease states, including cancer [31] and inflammatory conditions [100]. This led the current study to hypothesise that treatment with DHEA and DEM would lead to significant increases in the sensitivity of Kato III cells to SCFA treatment. However, it was demonstrated that combining propionate, but not butyrate, with the administration of these pharmacological agents, induced relatively small increases in cell death in this cancer cell type. This was an interesting finding, as it indicated the lack of any significant 'additive' effect on apoptosis-induction by the combination of these agents with SCFAs.

Potentially, these findings suggest that GSH availability may not play an important role in the modulation of apoptosis by SCFAs, at least in the Kato III cell line. However, as it has recently been proposed that butyrate may mediate its apoptotic effects, at least in part, through increasing oxidative stress [50], this seems very unlikely. It is proposed that results from the current study may be due to the capability of other cellular protective mechanisms, such as superoxide dismutase (SOD) and catalase, to scavenge ROS when GSH is unavailable [101, 102]. SOD and catalase are intracellular antioxidants that are known to reduce the superoxide anion ($^{\bullet}O_2^-$) and H_2O_2, respectively, in cooperation with GSH. We suggest, therefore, that when GSH availability was reduced by DHEA and DEM within the current study, SOD and catalase may have prevented any further increases in ROS production, thus preventing a further increase in oxidant-induced cell death. Future studies are required to assess SOD and catalase activity within this setting.

In conclusion, the current study has documented the ability of DEM and DHEA to effectively and immediately reduce GSH availability in Kato III gastric cancer cells. It was determined that

pre-incubation with DEM or DHEA significantly increased the sensitivity of Kato III cells to propionate but not butyrate. Moreover, DHEA treatment alone reduced cell viability to a greater extent than when in combination with propionate. It is proposed that future investigations could combine DEM and DHEA with current chemotherapeutic agents for the treatment of gastric and other human neoplasms.

CHAPTER 6: Short-chain fatty acid modulation of apoptosis and cell cycle regulation in Caco-2 human colon carcinoma cells

6.1: Introduction

Colorectal cancer is a major cause of cancer related death in the Western world [82, 103, 104]. It is estimated that up to 70% of colorectal cancers are caused by dietary factors and that a dietary approach could prevent much of this disease [103, 105]. Evidence is accumulating that the fermentation of complex carbohydrates and fibre by anaerobic bacteria, and the subsequent production of SCFAs, particularly butyrate, may play a major role in the modulatory effect of diet on colonic oncogenesis. Indeed, the effects of SCFAs in the colon are particularly important because the concentrations reported to trigger apoptosis, differentiation and cell cycle arrest are readily seen *in vivo* in the colonic lumen [43].

Previous studies have documented the induction of apoptosis by butyrate in colon cancer cell lines [11, 79], although very little is reported regarding the effects of a second SCFA, propionate, in this setting. Furthermore, some investigators have reported butyrate-induced cell cycle arrests in G0-G1 [43, 103, 106] while others have documented G2-M phase arrests [82]. Thus, debate exists regarding the regulatory effects of butyrate on the cell cycle that may be due to methodological differences between laboratories. Therefore, the present study investigated the capability of butyrate, propionate and a combination of both SCFAs, to induce apoptosis and cell cycle alterations in the human colon cancer cell line, Caco-2.

6.2: Materials and Methods

6.2.1: Materials

Caco-2 colon cancer cells were obtained from American Type Culture Collection (ATCC; Manassas, VA, USA; ATCC#: HTB-37). Dulbecco's modified Eagle's medium (DMEM), heat inactivated foetal bovine serum (FBS), Penicillin/Streptomycin (10,000units/ml, 10,000µg/ml), Dulbecco's phosphate buffered saline (DPBS) and Trypsin-EDTA were supplied by Invitrogen (Gibco®, Invitrogen Australia, VIC, Australia). Sodium butyrate and sodium propionate were obtained from Sigma-Aldrich (Castle Hill, NSW, Australia). Stock sodium butyrate, sodium propionate and a combination (1:1) of both were prepared using sterile water (200mM) and frozen at -20°C prior to the experiments. Annexin V FITC was obtained from Molecular Probes (Invitrogen Australia, VIC, Australia) and propidium iodide (PI) from Sigma-Aldrich.

6.2.2: Caco-2 cell culture

Caco-2 cells were cultured in 20ml complete DMEM medium containing 10% FBS and Penicillin/Streptomycin (90U/ml; 90µg/ml) in 75cm^2 cell culture flasks. Cells were maintained at 37°C in a humidified 5% CO_2 incubator.

6.2.3: Experimental design

Caco-2 cells were seeded into 6-well plates ($1.0x10^6$ viable cells/well) in 2ml complete DMEM medium and incubated for 72hr at 37°C prior to treatment. Pilot studies had shown that this cell number and duration of incubation allowed the formation of a complete monolayer containing approximately $3.5x10^6$ viable cells which is comparable to the cell number utilised within the Kato III treatment studies. Medium was replaced with fresh DMEM after 48hr incubation. Stock

sodium butyrate or sodium propionate (10µl, 50µl or 100µl) were then added to the wells in duplicate to give final concentrations of 1mM, 5mM or 10mM. A single concentration of the SCFA combination treatment was utilised (5mM, 50µl). Control wells received 50µl sterile water. Cells were incubated at 37°C and harvested after 24hr and 48hr prior to immediate assessment of cell viability and cell cycle as described below.

6.2.4: Cell harvesting

In order to effectively measure the induction of apoptosis and cell cycle alterations in SCFA-treated Caco-2 cells, both viable (attached) and non-viable (floating) cells were assessed. The present study devised a method for harvesting total Caco-2 cells as follows: Firstly, cell culture medium (2ml) containing floating, non-viable, cells was aliquoted into 10ml sterile tubes. Sterile DPBS (1ml) was added to individual wells, gently mixed and then aliquoted into the appropriate 10ml tube. Cells were washed once more with DPBS (1ml) and supernatant was discarded. Trypsin-EDTA (500µl) was then added to each well and plates were incubated for 5-8min at 37°C to allow for complete detachment of cells. Following this procedure, DMEM medium (1ml) was added to each well, mixed, aliquoted into eppendorf tubes (1.5ml) and centrifuged at 156 x g for 5min. Supernatant was removed and cells were resuspended in original medium containing non-viable cells thus producing a cell suspension containing both viable and non-viable cells. Aliquots of cell suspension (200µl) were added to flow-assisted cell sorting (FACS) tubes and assessed as described below.

6.2.5: Assays

6.2.5.1: Cell viability measurements

Caco-2 cell viability was assessed by flow cytometry as described in Chapter 3 (*3.2.4.1*). Cell viability was categorised into four classifications based on the amount of Annexin V FITC fluorescence and/or PI staining, as described previously: Viable (no Annexin V or PI), Total apoptosis (TA) which combined Late apoptotic (LA) cells (Annexin V and PI) and Early apoptotic (EA) cells (Annexin V only) or necrotic cells (PI only).

6.2.5.2: Cell cycle analysis

Alterations to cell cycle regulation in Caco-2 cells were assessed as described in Chapter 3 (*3.2.4.2*).

6.2.6: Statistics

All results are presented as mean ± SEM. Data were analysed using Two-way ANOVA's with Fisher's LSD *post-hoc* tests. Statistical analyses comparing differences in the ability of butyrate, propionate and the combination SCFA treatment to alter cell viability are only reported for comparisons of SCFAs between 5mM and 10mM and were analysed at individual time points using Kruskal-Wallis One-way ANOVA on Ranks with Student-Newman-Keuls *post-hoc* test. All statistics were calculated using SigmaStat 3.0 (SYSTAT Software Inc., California, USA). Significance was assumed at $p < 0.05$.

6.3: Results

6.3.1: Cell viability

6.3.1.1: Butyrate decreased cell viability and induced apoptosis in Caco-2 cells

Incubation with butyrate significantly reduced the viability of Caco-2 cells undergoing apoptosis after 24hr (1mM, $p < 0.001$, $n = 6$; 5mM, $p < 0.001$, $n = 6$; 10mM, $p < 0.001$, $n = 6$) and 48hr (1mM, $p < 0.001$, $n = 6$; 5mM, $p < 0.001$, $n = 6$; 10mM, $p < 0.001$, $n = 6$) compared to vehicle control (Table 6.1). At both time points, Caco-2 viability was significantly less following incubation with 5mM ($p < 0.001$) and 10mM ($p < 0.001$) butyrate than with 1mM butyrate.

The proportion of TA in Caco-2 cells was significantly increased after 24hr (1mM, $p < 0.001$; 5mM, $p < 0.001$; 10mM, $p < 0.001$) and 48hr (1mM, $p < 0.001$; 5mM, $p < 0.001$; 10mM, $p < 0.001$) compared to control (Table 6.1). No differences were observed between each concentration at 24hr ($p > 0.05$) however, after 48hr incubation, 5mM ($p < 0.001$) and 10mM ($p < 0.001$) butyrate induced significantly greater TA than 1mM butyrate.

The percentage of Caco-2 cells undergoing LA was significantly increased after 24hr (1mM, $p < 0.001$; 5mM, $p < 0.001$; 10mM, $p < 0.001$) and 48hr (1mM, $p < 0.001$; 5mM, $p < 0.001$; 10mM, $p < 0.001$) compared to control (Table 6.1). LA was induced to a greater extent after 48hr incubation with 5mM ($p < 0.001$) and 10mM ($p < 0.001$) butyrate than 1mM butyrate but not at 24hr incubation.

Table 6.1: Percentage Caco-2 cell viability after incubation with butyrate

Cell viability classification	Concentration of butyrate (mM)	Duration of treatment (hr) 24	48
Viable	**0**	76.4 ± 0.9	70.8 ± 1.1
	1	61.8 ± 0.6[c]	61.0 ± 0.2[c]
	5	53.6 ± 2.0[cd]	24.8 ± 0.5[cd]
	10	55.0 ± 1.9[cd]	22.3 ± 0.9[cd]
Total apoptotic	**0**	11.4 ± 0.5	18.5 ± 0.5
	1	25.5 ± 1.1[c]	26.5 ± 0.7[c]
	5	27.3 ± 2.2[c]	54.1 ± 1.9[cd]
	10	24.5 ± 2.7[c]	51.1 ± 1.5[cd]
Late apoptotic	**0**	8.5 ± 0.4	14.0 ± 0.4
	1	19.8 ± 0.9[c]	21.2 ± 0.5[c]
	5	22.2 ± 2.0[c]	41.3 ± 0.9[cd]
	10	19.5 ± 2.5[c]	40.9 ± 1.4[cd]
Early apoptotic	**0**	2.9 ± 0.1	4.7 ± 0.3
	1	5.7 ± 0.3[c]	5.3 ± 0.3
	5	5.2 ± 0.3[c]	12.7 ± 1.4[cd]
	10	4.9 ± 0.3	10.2 ± 1.4[cde]
Necrotic	**0**	12.2 ± 1.3	10.6 ± 1.0
	1	12.7 ± 0.9	12.5 ± 0.8
	5	19.1 ± 0.8[cd]	21.1 ± 1.9[cd]
	10	20.5 ± 1.6[cd]	26.6 ± 1.6[cde]

Data are presented as mean (%) ± SEM (n = 6). All treatment groups were compared to control ([c]$p < 0.001$), 1mM butyrate ([d]$p < 0.05$) or 5mM butyrate ([e]$p < 0.05$).

An increase in the proportion of Caco-2 cells undergoing EA with butyrate treatment was observed after 24hr (1mM, $p < 0.05$; 5mM, $p < 0.05$) and 48hr (5mM, $p < 0.001$; 10mM, $p < 0.001$) incubation compared to control (Table 6.1). A greater induction of EA was apparent after 48hr incubation with 5mM ($p < 0.001$) and 10mM ($p < 0.001$) butyrate compared to 1mM butyrate.

The percentage of Caco-2 cells undergoing necrosis was significantly increased after 24hr (5mM, $p < 0.001$; 10mM, $p < 0.001$) and 48hr (5mM, $p < 0.001$; 10mM, $p < 0.001$) incubation with butyrate compared to vehicle control (Table 6.1). The proportion of Caco-2 cells undergoing necrosis was greater after 24hr incubation with 5mM ($p < 0.01$) and 10mM ($p < 0.001$) butyrate compared to 1mM butyrate. After 48hr incubation, the percentage of necrotic Caco-2 cells was significantly increased following incubation with 5mM ($p < 0.001$) and 10mM ($p < 0.001$) butyrate compared to control. A greater percentage of necrotic cells were observed after 48hr incubation with 5mM ($p < 0.001$) and 10mM ($p < 0.001$) compared to 1mM butyrate. Additionally, 10mM butyrate induced a greater percentage of necrosis compared to treatment with 5mM butyrate ($p < 0.01$).

6.3.1.2: Propionate reduced viability and increased apoptosis in Caco-2 cells

The proportion of viable Caco-2 cells was significantly reduced after 24hr (1mM, $p < 0.001$, $n = 6$; 5mM, $p < 0.001$, $n = 6$; 10mM, $p < 0.001$, $n = 6$) and 48hr (5mM, $p < 0.001$, $n = 6$; 10mM, $p < 0.001$, $n = 6$) incubation with propionate compared to vehicle control values (Table 6.2). A greater decrease in Caco-2 viability was apparent after 24hr and 48hr incubation with 5mM ($p < 0.001$) and 10mM ($p < 0.001$) propionate than 1mM propionate. Incubation with 10mM propionate resulted in greater reductions in viability after 48hr incubation than with 5mM propionate ($p < 0.001$).

Incubation with propionate significantly increased the percentage if TA in Caco-2 cells after 24hr (5mM, $p < 0.001$; 10mM, $p < 0.001$) and 48hr (5mM, $p < 0.001$; 10mM, $p < 0.001$) compared to control untreated cells (Table 6.2). After 24hr and 48hr incubation, both 5mM ($p < 0.001$) and 10mM ($p < 0.001$) propionate increased TA significantly greater than 1mM propionate. In addition, 10mM ($p < 0.001$) propionate induced greater TA than 5mM propionate after 48hr incubation.

The percentage of Caco-2 cells undergoing LA was significantly increased after 24hr (1mM, $p < 0.05$; 5mM, $p < 0.001$; 10mM, $p < 0.001$) and 48hr (5mM, $p < 0.001$; 10mM, $p < 0.001$) incubation with propionate compared to vehicle control (Table 6.2). A greater increase in LA was observed after 24hr and 48hr incubation with 5mM ($p < 0.001$) and 10mM ($p < 0.001$) propionate compared to 1mM propionate. After 48hr incubation, 10mM propionate induced greater LA than the 5mM concentration of propionate ($p < 0.001$).

Propionate did not alter the percentage of Caco-2 cells undergoing EA after 24hr at any concentration ($p > 0.05$) (Table 6.2). However, after 48hr, 10mM propionate increased EA compared to 1mM ($p < 0.05$) and 5mM ($p < 0.01$) propionate but not compared to control ($p > 0.05$).

Table 6.2: Percentage Caco-2 cell viability after incubation with propionate

Cell viability classification	Concentration of butyrate (mM)	Duration of treatment (hr) 24	48
Viable	**0**	76.4 ± 0.9	70.8 ± 1.1
	1	69.3 ± 0.9^{c}	69.1 ± 0.7
	5	59.9 ± 0.9^{cd}	58.6 ± 0.5^{cd}
	10	58.4 ± 0.3^{cd}	44.6 ± 0.3^{cde}
Total apoptotic	**0**	11.4 ± 0.5	18.5 ± 0.5
	1	14.4 ± 0.7	19.3 ± 0.4
	5	23.9 ± 2.3^{cd}	26.1 ± 1.2^{cd}
	10	21.8 ± 1.0^{cd}	34.3 ± 0.8^{cde}
Late apoptotic	**0**	8.5 ± 0.4	14.0 ± 0.4
	1	12.0 ± 0.5^{c}	14.8 ± 0.6
	5	20.8 ± 2.1^{cd}	21.9 ± 0.8^{cd}
	10	18.6 ± 0.7^{cd}	28.7 ± 0.6^{cde}
Early apoptotic	**0**	2.9 ± 0.1	4.7 ± 0.3
	1	2.4 ± 0.2	4.5 ± 0.5
	5	3.2 ± 0.3	4.1 ± 0.5
	10	3.2 ± 0.3	5.6 ± 0.2^{de}
Necrotic	**0**	12.2 ± 1.3	10.6 ± 1.0
	1	16.3 ± 1.0^{a}	11.7 ± 0.7
	5	16.1 ± 1.8^{a}	15.3 ± 1.5^{cd}
	10	19.8 ± 0.9^{cde}	21.1 ± 0.6^{cde}

Data are presented as mean (%) ± SEM (n = 6). All treatment groups were compared to control ($^{a}p < 0.05$, $^{b}p < 0.01$, $^{c}p < 0.001$), 1mM butyrate ($^{d}p < 0.05$) or 5mM butyrate ($^{e}p < 0.05$).

Incubation with propionate increased the proportion of necrotic Caco-2 cells after 24hr (1mM, $p < 0.05$; 5mM, $p < 0.05$; 10mM, $p < 0.001$) and 48hr (5mM, $p < 0.01$; 10mM, $p < 0.001$) compared to vehicle control values (Table 6.2). A greater increase in necrosis was observed after 24hr incubation with 10mM propionate compared to 5mM ($p < 0.05$) and 1mM ($p < 0.05$) propionate. After 48hr incubation, the percentage of necrotic Caco-2 cells was greater after incubation with 5mM propionate than 1mM propionate ($p < 0.05$).

6.3.1.3: Combination SCFA treatment reduced viability and increased apoptosis in Caco-2 cells

Incubation with 5mM butyrate/propionate mix significantly reduced Caco-2 cell viability after 24hr ($p < 0.001$) and 48hr ($p < 0.001$) compared to control (Table 6.3). The percentage of TA in Caco-2 cells was increased after 24hr ($p < 0.001$) and 48hr ($p < 0.001$) incubation with SCFA combination treatment compared to vehicle control. An increase in LA was observed in Caco-2 cells after 24hr ($p < 0.001$) and 48hr ($p < 0.001$) incubation with 5mM butyrate/propionate mix compared to control values. The percentage of Caco-2 cells undergoing EA was significantly increased after 24hr ($p < 0.001$) and 48hr ($p < 0.01$) incubation with 5mM SCFA combination treatment compared to control. A significant increase in the proportion of necrotic Caco-2 cells was observed after 24hr ($p < 0.001$) and 48hr ($p < 0.001$) incubation with SCFA combination treatment than control untreated cells (Table 6.3).

Table 6.3: Percentage viability of Caco-2 cells following incubation with 5mM SCFA combination treatment.

Cell viability classification	Concentration of SCFA mix (mM)	Duration of treatment (hr) 24	48
Viable	**0**	76.4 ± 0.9	70.8 ± 1.1
	5	51.6 ± 0.5[c]	25.3 ± 0.3[c]
Total apoptotic	**0**	11.4 ± 0.5	18.5 ± 0.5
	5	26.5 ± 0.8[c]	49.5 ± 1.3[c]
Late apoptotic	**0**	8.5 ± 0.4	14.0 ± 0.4
	5	22.4 ± 0.6[c]	41.7 ± 0.7[c]
Early apoptotic	**0**	2.9 ± 0.1	4.7 ± 0.3
	5	4.2 ± 0.2[c]	7.9 ± 0.6[b]
Necrotic	**0**	12.2 ± 1.3	10.6 ± 1.0
	5	21.9 ± 0.7[c]	25.2 ± 1.5[c]

Data are presented as mean (%) ± SEM (n = 6). All treatment groups were compared to control ([b]$p < 0.01$, [c]$p < 0.001$).

6.3.2: Comparisons of viability and cell death between all treatment groups

6.3.2.1: Butyrate reduced viability and increased apoptosis greater than propionate alone or SCFA combination treatment

Incubation with butyrate decreased Caco-2 cell viability greater than equivalent concentrations of propionate after 24hr ($p < 0.05$) and 48hr ($p < 0.05$) incubation (Figure 6.1). Caco-2 cell viability was significantly lower following 24hr incubation with 5mM SCFA combination treatment than butyrate (5mM, $p < 0.05$; 10mM, $p < 0.05$) alone and propionate (5mM, $p < 0.05$; 10mM, $p < 0.05$) alone. Viability was significantly less after 48hr incubation with 5mM SCFA combination treatment than after propionate treatment (5mM, $p < 0.05$; 10mM, $p < 0.05$) but not after 5mM butyrate treatment ($p < 0.05$). However, 48hr incubation with 10mM butyrate reduced cell viability greater ($p < 0.05$) than 5mM SCFA combination treatment (Figure 6.1).

After 24hr incubation, 1mM butyrate increased the percentage of TA greater than 1mM propionate ($p < 0.05$) (Figure 6.2). Incubation with butyrate for 48hr increased TA greater than propionate treatment at all equivalent concentrations ($p < 0.05$). No differences in TA were observed between butyrate treatment alone and 5mM SCFA combination treatment ($p < 0.05$) after 48hr incubation. In contrast, 5mM SCFA combination treatment increased TA greater than all propionate concentrations ($p < 0.05$) (Figure 6.2).

After 24hr incubation with 1mM butyrate, LA was increased greater than 1mM propionate ($p < 0.05$) (Figure 6.3). Following 48hr incubation, LA was increased the greatest after treatment with butyrate than propionate at all equivalent concentrations ($p < 0.05$). No differences in the percentage of LA were observed after 48hr incubation with 5mM SCFA combination treatment and butyrate treatment (5mM, $p > 0.05$; 10mM, $p > 0.05$). However, all concentrations of propionate induced less LA than 5mM SCFA combination treatment ($p < 0.05$) (Figure 6.3).

The percentage of EA with butyrate was significantly greater after 24hr and 48hr incubation compared to propionate at all equivalent concentrations ($p < 0.05$) (Figure 6.4). SCFA combination treatment induced less EA in Caco-2 cells than 5mM butyrate ($p < 0.05$) but more than propionate (5mM, $p < 0.05$; 10mM, $p < 0.05$) treatment. After 48hr incubation, 5mM SCFA combination treatment induced less EA than butyrate treatment (5mM, $p < 0.05$; 10mM, $p < 0.05$) alone but significantly greater than propionate treatment (5mM, $p < 0.05$; 10mM, $p < 0.05$) alone (Figure 6.4).

The percentage of necrotic Caco-2 cells was greater after 24hr incubation with 5mM SCFA combination treatment than 5mM propionate alone ($p < 0.05$) but not with any other treatments (Figure 6.5). Incubation with 5mM butyrate increased necrosis in Caco-2 cells greater than 5mM propionate ($p < 0.05$) after 48hr incubation. Additionally, 5mM SCFA combination treatment increased necrosis in Caco-2 cells greater than 5mM propionate ($p < 0.05$) (Figure 6.5).

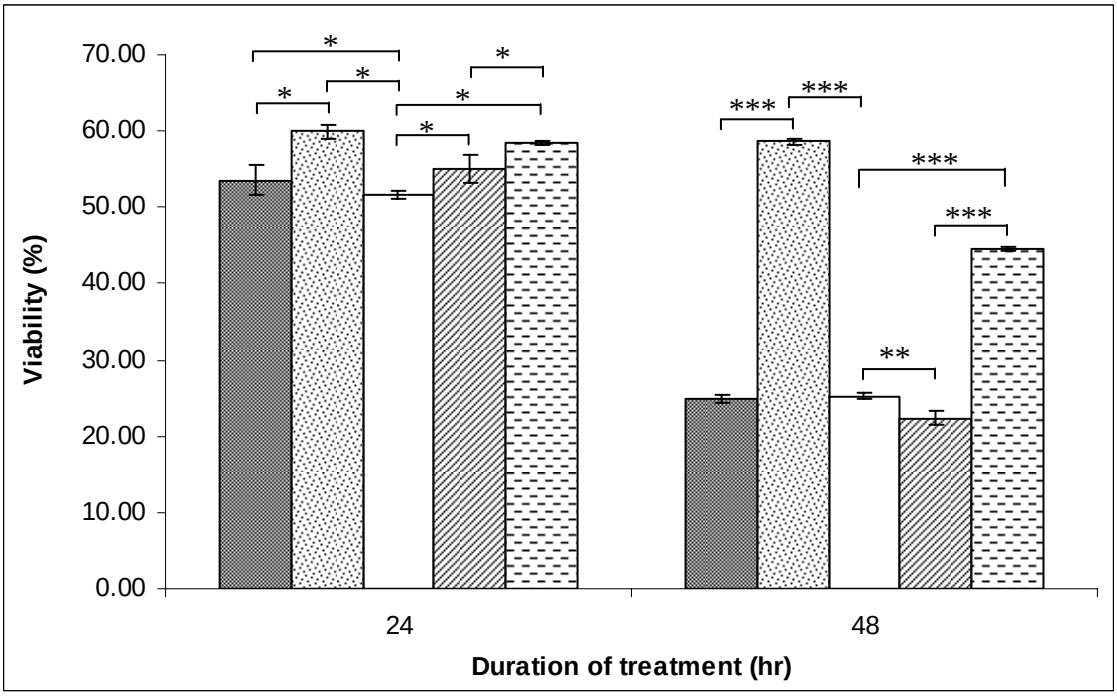

Figure 6.1: A comparison of the viability of Caco-2 cells following incubation with 5mM butyrate ▩, 5mM propionate ▨, 5mM SCFA combination □, 10mM butyrate ▨ and 10mM propionate ▨. Data are presented as mean ± SEM (n = 6). *p < 0.05, **p < 0.01, ***p < 0.001.

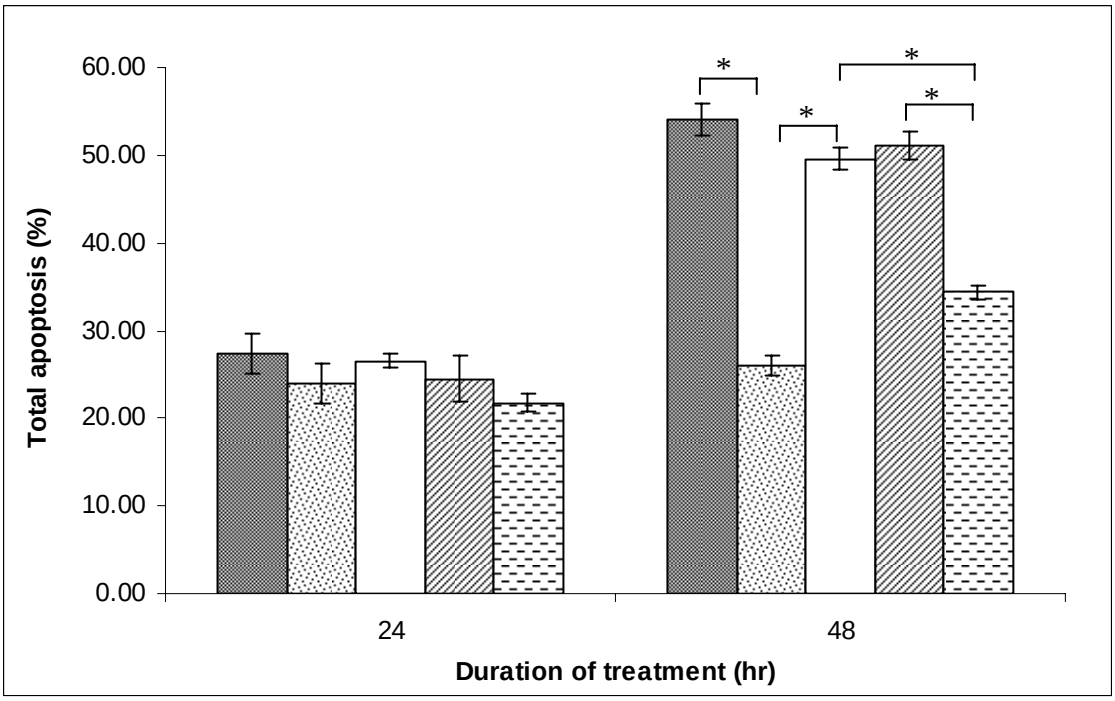

Figure 6.2: A comparison of the proportion of total apoptosis in Caco-2 cells following incubation with 5mM butyrate ▩, 5mM propionate ▩, 5mM SCFA combination □, 10mM butyrate ▨ and 10mM propionate ▤. Data are presented as mean ± SEM (n = 6). *$p < 0.05$.

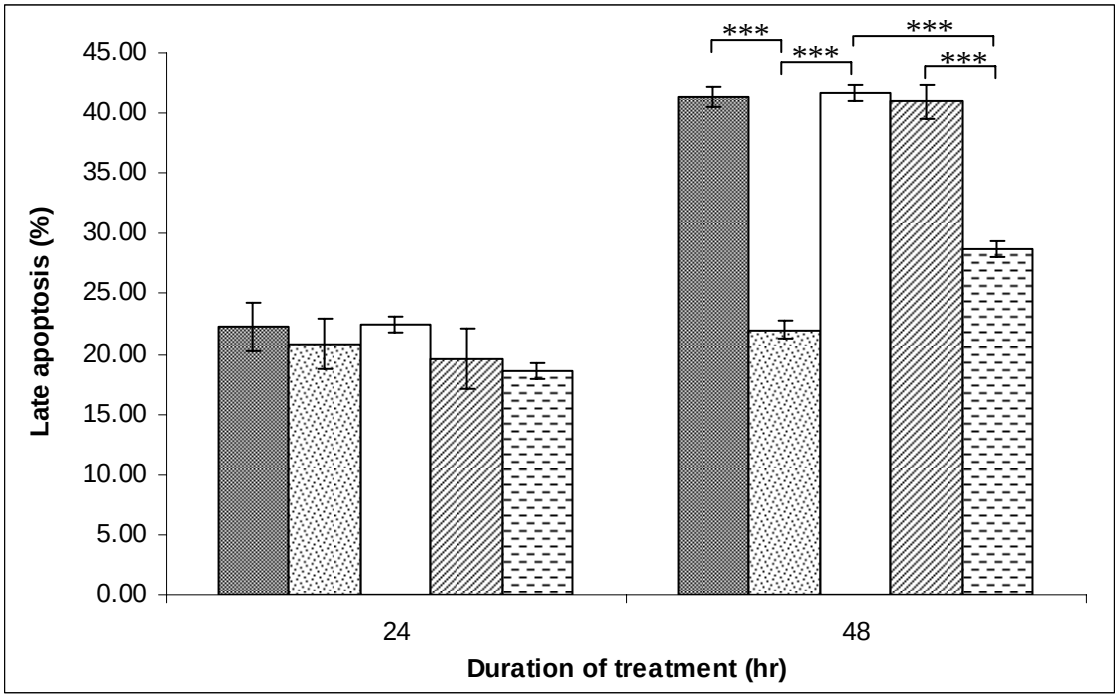

Figure 6.3: A comparison of the proportion of late apoptosis in Caco-2 cells following incubation with 5mM butyrate ▩, 5mM propionate ▩, 5mM SCFA combination □, 10mM butyrate ▨ and 10mM propionate ▨. Data are presented as mean ± SEM (n = 6). ***p < 0.001.

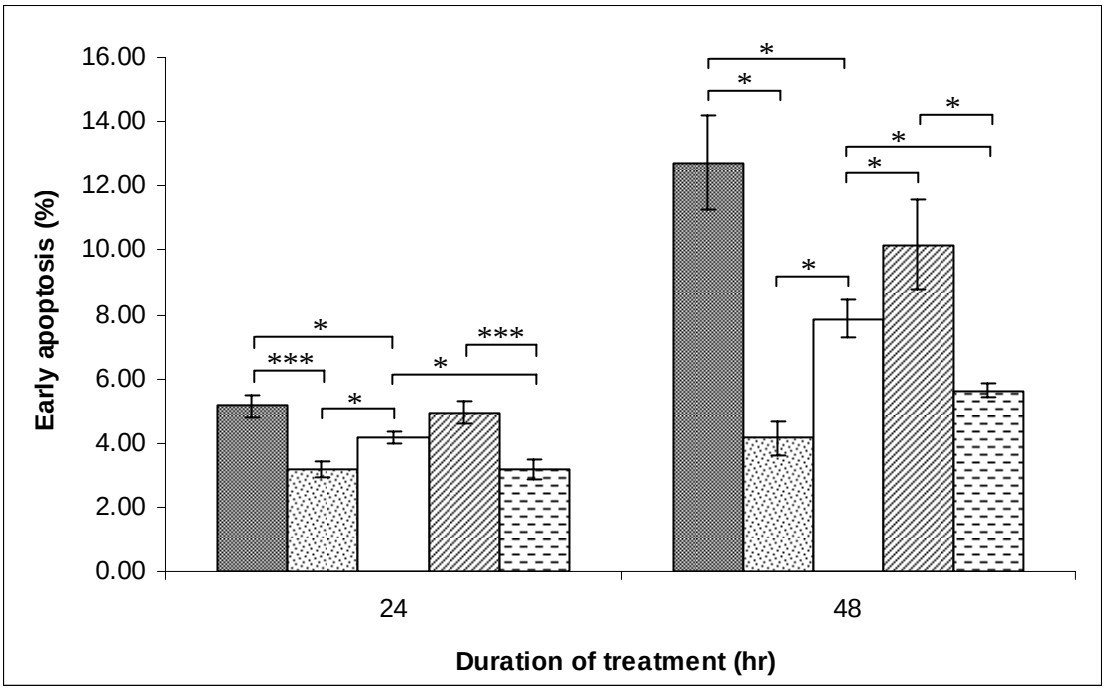

Figure 6.4: A comparison of the proportion of early apoptosis in Caco-2 cells following incubation with 5mM butyrate ▩, 5mM propionate ▦, 5mM SCFA combination □, 10mM butyrate ▨ and 10mM propionate ▤. Data are presented as mean ± SEM (n = 6). *$p < 0.05$, ***$p < 0.001$.

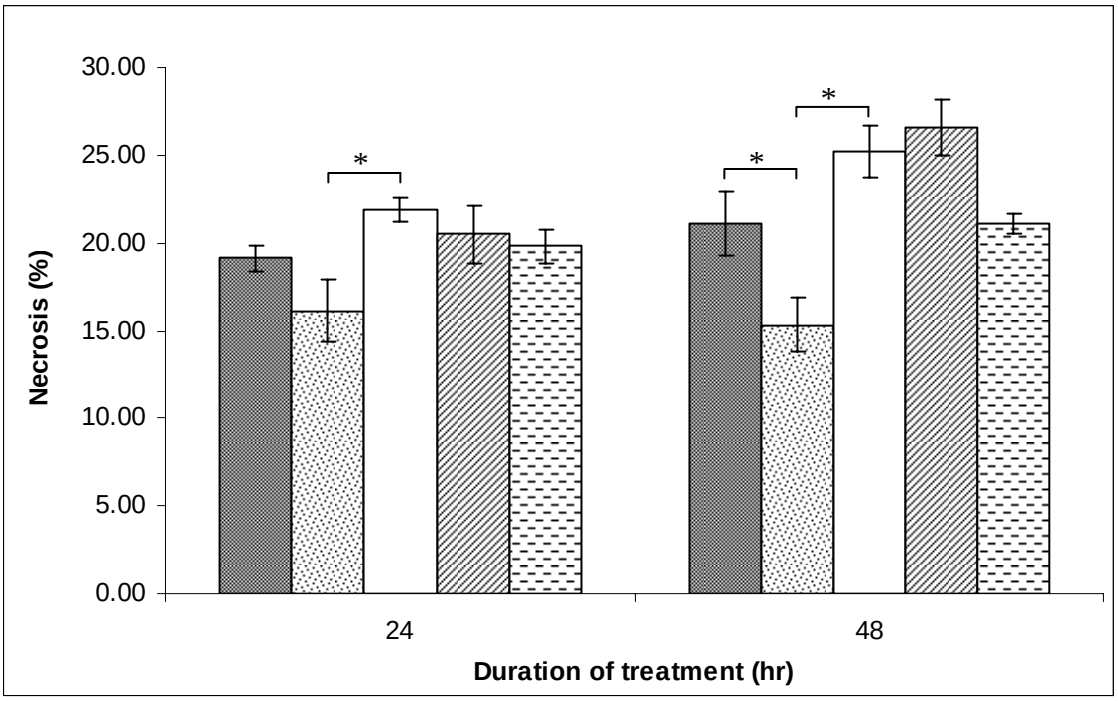

Figure 6.5: A comparison of the proportion of necrotic Caco-2 cells following incubation with 5mM butyrate ▩, 5mM propionate ▦, 5mM SCFA combination □, 10mM butyrate ▨ and 10mM propionate ▤. Data are presented as mean ± SEM (n = 6). *$p < 0.05$.

6.3.3: Cell cycle results

6.3.3.1: Butyrate treatment reduced the percentage of Caco-2 cells in G0-G1 phase

Incubation with butyrate decreased the percentage of Caco-2 cells in G0-G1 phase after 24hr (1mM, $p < 0.001$, $n = 6$; 5mM, $p < 0.001$, $n = 6$; 10mM, $p < 0.001$, $n = 6$) and 48hr (1mM, $p < 0.001$, $n = 6$; 5mM, $p < 0.001$, $n = 6$; 10mM, $p < 0.001$, $n = 6$) compared to vehicle controls (Table 6.4). A greater proportion of Caco-2 cells were observed in G0-G1 phase after 24hr and 48hr incubation with 10mM ($p < 0.001$) and 5mM ($p < 0.001$) than incubation with 1mM butyrate. Also, 48hr incubation with 10mM butyrate reduced the proportion of Caco-2 cells in G0-G1 phase greater than 5mM ($p < 0.001$) butyrate.

6.3.3.2: Butyrate treatment increased the percentage of Caco-2 cells in S-phase

The percentage of Caco-2 cell in S-phase was increased after 24hr incubation with butyrate (1mM, $p < 0.001$, $n = 6$; 5mM, $p < 0.001$, $n = 6$; 10mM, $p < 0.001$, $n = 6$) compared to controls (Table 6.4). At this time point, incubation with 10mM butyrate induced a greater increase in S-phase Caco-2 cells than 1mM ($p < 0.001$) and 5mM ($p < 0.001$) butyrate. Additionally, 5mM butyrate induced a greater proportion of Caco-2 cells into S-phase than 1mM butyrate ($p < 0.001$). After 48hr, a decrease in the number of cells undergoing S-phase was observed after incubation with 1mM butyrate ($p < 0.05$) and an increase after incubation with 10mM butyrate compared to controls. No differences were observed after incubation with 5mM butyrate compared to control values (Table 6.4).

6.3.3.3: Butyrate treatment increased the percentage of Caco-2 cells in G2-M phase

Butyrate treatment significantly increased the percentage of Caco-2 cells in G2-M phase after 24hr (1mM, $p < 0.001$; 5mM, $p < 0.001$; 10mM, $p < 0.001$) and 48hr (1mM, $p < 0.001$; 5mM, $p < 0.001$; 10mM, $p < 0.001$) compared to vehicle control (Table 6.4). Incubation with 10mM

butyrate induced a greater increase in G2-M phase Caco-2 cells than 1mM butyrate ($p < 0.001$) but less than 5mM butyrate ($p < 0.001$) after 24hr.

Incubation with 5mM butyrate increased G2-M phase cells greater than 1mM butyrate ($p < 0.001$) after 24hr (Table 6.4). In addition, 10mM butyrate induced a greater proportion of Caco-2 cells to enter G2-M phase than 5mM ($p < 0.001$) and 1mM ($p < 0.001$) butyrate while 5mM butyrate also caused a greater number to enter G2-M phase than 1mM butyrate ($p < 0.001$) after 48hr incubation.

6.3.3.4: Propionate reduced the percentage of Caco-2 cells in G0-G1 phase

A significant reduction in the proportion of Caco-2 cells in G0-G1 phase was observed after 24hr (5mM, $p < 0.001$; 10mM, $p < 0.001$) and 48hr (5mM, $p < 0.001$; 10mM, $p < 0.001$) incubation with propionate compared to vehicle controls (Table 6.5). However, 1mM propionate increased the number of Caco-2 cells in G0-G1 phase after 48hr incubation compared to control. Incubation with 5mM and 10mM butyrate reduced the percentage of Caco-2 cells in G0-G1 greater than 1mM butyrate ($p < 0.001$) at both 24hr and 48hr. At both time points, 10mM butyrate also reduced G0-G1 phase Caco-2 cells greater than 5mM butyrate ($p < 0.001$) (Table 6.5).

Table 6.4: Cell cycle results in Caco-2 cells following incubation with butyrate

Cell cycle phase	Concentration of butyrate (mM)	Duration of treatment (hr) 24	48
G0-G1	**0**	67.1 ± 0.4	70.3 ± 0.5
	1	57.6 ± 0.2[c]	68.1 ± 0.6[c]
	5	48.6 ± 0.5[cd]	50.5 ± 0.6[cd]
	10	49.5 ± 0.2[cd]	48.2 ± 0.3[cde]
S	**0**	13.5 ± 0.2	11.6 ± 0.5
	1	16.5 ± 0.2[c]	11.1 ± 0.2[c]
	5	19.3 ± 0.3[cd]	11.8 ± 0.3
	10	21.8 ± 0.2[cde]	12.6 ± 0.2[c]
G2-M	**0**	17.6 ± 0.9	17.3 ± 0.3
	1	24.4 ± 0.2[c]	19.8 ± 0.5[c]
	5	30.4 ± 0.5[cd]	35.4 ± 0.7[cd]
	10	26.9 ± 0.3[cde]	36.9 ± 0.4[cde]

Data are presented as mean (%) ± SEM (n = 6). All treatment groups were compared to control ([c]$p < 0.001$), 1mM butyrate ([d]$p < 0.05$) or 5mM butyrate ([e]$p < 0.05$).

Table 6.5: Cell cycle results in Caco-2 cells following incubation with propionate

Cell cycle phase	Concentration of propionate (mM)	Duration of treatment (hr) 24	48
G0-G1	**0**	67.1 ± 0.4	70.3 ± 0.5
	1	66.9 ± 0.3	73.1 ± 0.7[c]
	5	61.3 ± 0.4[cd]	62.2 ± 0.4[cd]
	10	58.7 ± 0.3[cde]	53.4 ± 0.7[cde]
S	**0**	13.5 ± 0.2	11.6 ± 0.5
	1	14.9 ± 0.1[c]	11.1 ± 0.2
	5	16.9 ± 0.2[cd]	13.1 ± 0.2[cd]
	10	18.1 ± 0.1[cde]	13.9 ± 0.4[cde]
G2-M	**0**	17.6 ± 0.9	17.3 ± 0.3
	1	17.0 ± 0.3	14.7 ± 0.5[c]
	5	20.2 ± 0.2[cd]	23.7 ± 0.2[cd]
	10	21.6 ± 0.2[cd]	31.2 ± 0.6[cde]

Data are presented as mean (%) ± SEM (n = 6). All treatment groups were compared to control ([c]$p < 0.001$), 1mM butyrate ([d]$p < 0.05$) or 5mM butyrate ([e]$p < 0.05$).

6.3.3.5: Propionate increased the percentage of Caco-2 cells in S-phase

Incubation with propionate increased the percentage of Caco-2 cells in S-phase after 24hr (1mM, $p < 0.001$, $n = 6$; 5mM, $p < 0.001$, $n = 6$; 10mM, $p < 0.001$, $n = 6$) and 48hr (5mM, $p < 0.001$, $n = 6$; 10mM, $p < 0.001$, $n = 6$) compared to vehicle control cells (Table 6.5). After both 24hr and 48hr incubation, 5mM and 10mM propionate induced a greater increase in the proportion of Caco-2 cells in S-phase than 1mM propionate ($p < 0.001$) while 10mM also caused a greater increase than 5mM propionate after 24hr ($p < 0.01$) and 48hr ($p < 0.05$).

6.3.3.6: Propionate treatment increased the proportion of Caco-2 cells in G2-M phase

The percentage of Caco-2 cells in G2-M phase was significantly increased after 24hr (5mM, $p < 0.001$, $n = 6$; 10mM, $p < 0.001$, $n = 6$) and 48hr (5mM, $p < 0.001$, $n = 6$; 10mM, $p < 0.001$, $n = 6$) incubation with propionate compared to control (Table 6.5). However, a decrease was observed after 48hr incubation with 1mM propionate ($p < 0.001$) compared to control. Incubation with 10mM and 5mM propionate induced a greater proportion of Caco-2 cells to enter G2-M phase than 1mM ($p < 0.001$) propionate at each time point. In addition, after 48hr incubation, 5mM propionate induced a greater increase in the number of Caco-2 cells in G2-M phase than 5mM propionate ($p < 0.001$).

6.3.3.7: SCFA combination treatment increased the percentage of Caco-2 cells in G2-M phase

Incubation with the SCFA combination treatment (5mM butyrate/propionate) significantly decreased the proportion of Caco-2 cells in G0-G1 phase after 24hr ($p < 0.001$, $n = 6$) and 48hr ($p < 0.001$, $n = 6$) compared to vehicle control (Table 6.6). The percentage of Caco-2 cells in S phase was increased after 24hr ($p < 0.001$) incubation with the SCFA combination treatment but not after 48hr ($p > 0.05$) incubation compared to control. An increase in the percentage of Caco-2

cells in G2-M phase was observed after 24hr ($p < 0.05$) and 48hr ($p < 0.001$) incubation with the 5mM SCFA combination treatment (Table 6.6).

Table 6.6: Cell cycle results in Caco-2 cells following incubation with 5mM SCFA combination treatment

Cell cycle phase	Concentration of SCFA mix (mM)	Duration of treatment (hr) 24	48
G0-G1	**0**	67.1 ± 0.4	70.3 ± 0.5
	5	56.3 ± 1.0[c]	49.5 ± 0.7[c]
S	**0**	13.5 ± 0.2	11.6 ± 0.5
	5	19.4 ± 0.5[c]	12.1 ± 0.3
G2-M	**0**	17.6 ± 0.9	17.3 ± 0.3
	5	21.3 ± 1.2[a]	36.4 ± 0.5[c]

Data are presented as mean (%) ± SEM (n = 6). All treatment groups were compared to control ([a]$p < 0.05$, [c]$p < 0.001$).

6.3.4: Comparisons of cell cycle results between all treatment groups

6.3.4.1: Butyrate alone and SCFA combination treatment reduced the percentage of Caco-2 cells in G0-G1 greater than propionate alone

Butyrate treatment reduced the percentage of Caco-2 cells in G0-G1 phase greater than propionate after 24hr and 48hr incubation at all equivalent concentrations ($p < 0.01$) (Figure 6.6). SCFA combination treatment reduced G0-G1 phase cells greater than 5mM and 10mM propionate after 24hr (5mM, $p < 0.001$; 10mM, $p < 0.01$) and 48hr (5mM, $p < 0.001$; 10mM, $p < 0.001$). However, SCFA combination treatment did not reduce the number of Caco-2 cells in G0-G1 phase to the extent of 5mM ($p < 0.001$) or 10mM butyrate ($p < 0.001$) after 24hr incubation. No differences were observed in the percentage of Caco-2 cells in G0-G1 phase following 48hr treatment 5mM and 10mM butyrate compared to 5mM SCFA combination treatment ($p > 0.05$) (Figure 6.6).

6.3.4.2: SCFA treatments initially increased and then decreased the percentage of Caco-2 cells in S-phase

After 24hr incubation, butyrate increased the percentage of Caco-2 cells in S-phase greater than equivalent concentrations of propionate (1mM, $p < 0.001$; 5mM, $p < 0.001$; 10mM, $p < 0.001$) (Figure 6.7). SCFA combination treatment induced greater increases in the number of S-phase Caco-2 cells than 5mM propionate ($p < 0.001$) and 10mM propionate ($p < 0.01$) alone after 24hr. However, 10mM butyrate significantly induced a greater percentage of Caco-2 cells into S-phase ($p < 0.001$) than 5mM SCFA combination treatment. The percentage of Caco-2 cells remaining in S-phase after 48hr was significantly greater with propionate treatment than incubation with equivalent concentrations of butyrate (5mM, $p < 0.05$; 10mM, $p < 0.01$) alone and 5mM SCFA combination treatment ($p < 0.01$) (Figure 6.7).

6.3.4.3: Butyrate alone and SCFA combination treatment induced Caco-2 cells to enter G2-M greater than propionate alone

All concentrations of butyrate induced a greater percentage of Caco-2 cells to enter G2-M phase than equivalent concentrations of propionate alone after 24hr (1mM, $p < 0.05$; 5mM, $p < 0.05$; 10mM, $p < 0.05$) and 48hr incubation (1mM, $p < 0.001$; 5mM, $p < 0.001$; 10mM, $p < 0.001$) (Figure 6.8). It was also observed that 24hr incubation with 1mM and 5mM butyrate induced a greater percentage of Caco-2 cells to enter G2-M phase than 5mM and 10mM propionate ($p < 0.05$), respectively. The SCFA combination treatment induced significantly less Caco-2 cells into G2-M phase than 5mM and 10mM butyrate treatment alone ($p < 0.05$) after 24hr incubation but showed no differences to butyrate treatment alone ($p > 0.05$). No differences in the proportion of Caco-2 cells within G2-M phase were observed between those treated with butyrate alone or SCFA combination treatment ($p > 0.05$). However, 5mM and 10mM propionate caused less Caco-2 cells to enter G2-M phase than 5mM SCFA combination treatment ($p < 0.001$) (Figure 6.8).

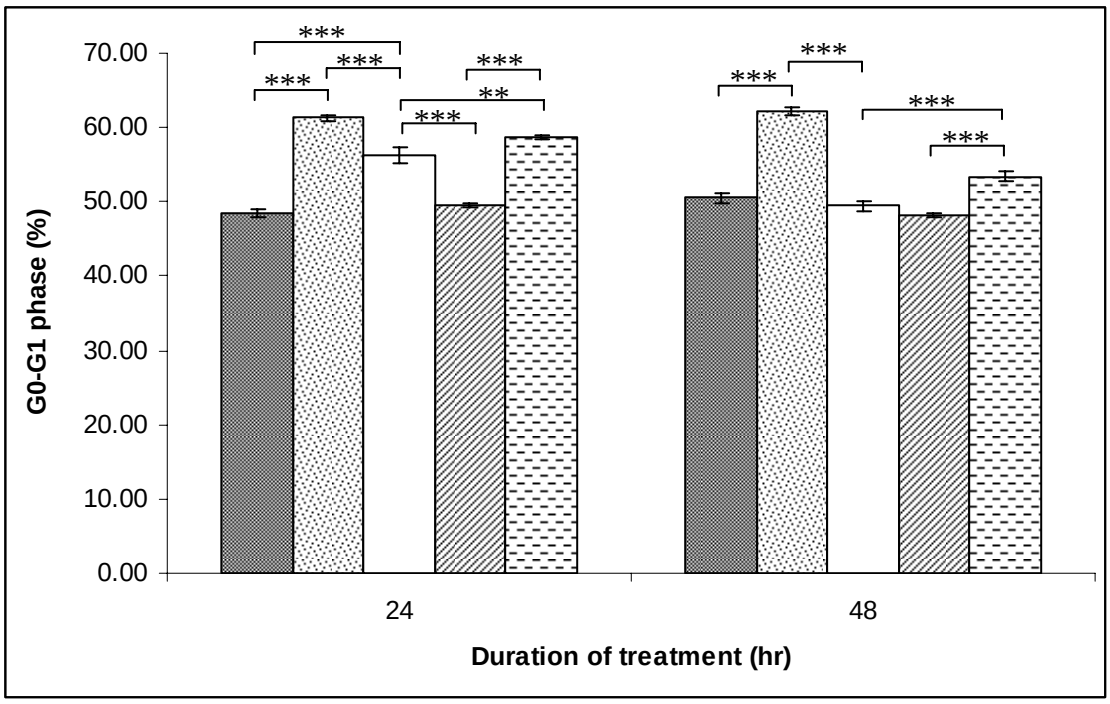

Figure 6.6: A comparison of the proportion of G0-G1 phase Caco-2 cells following incubation with 5mM butyrate ▩, 5mM propionate ⬚, 5mM SCFA combination□, 10mM butyrate▨ and 10mM propionate ⬚. Data are presented as mean ± SEM (n = 6). **p < 0.01, ***p < 0.001.

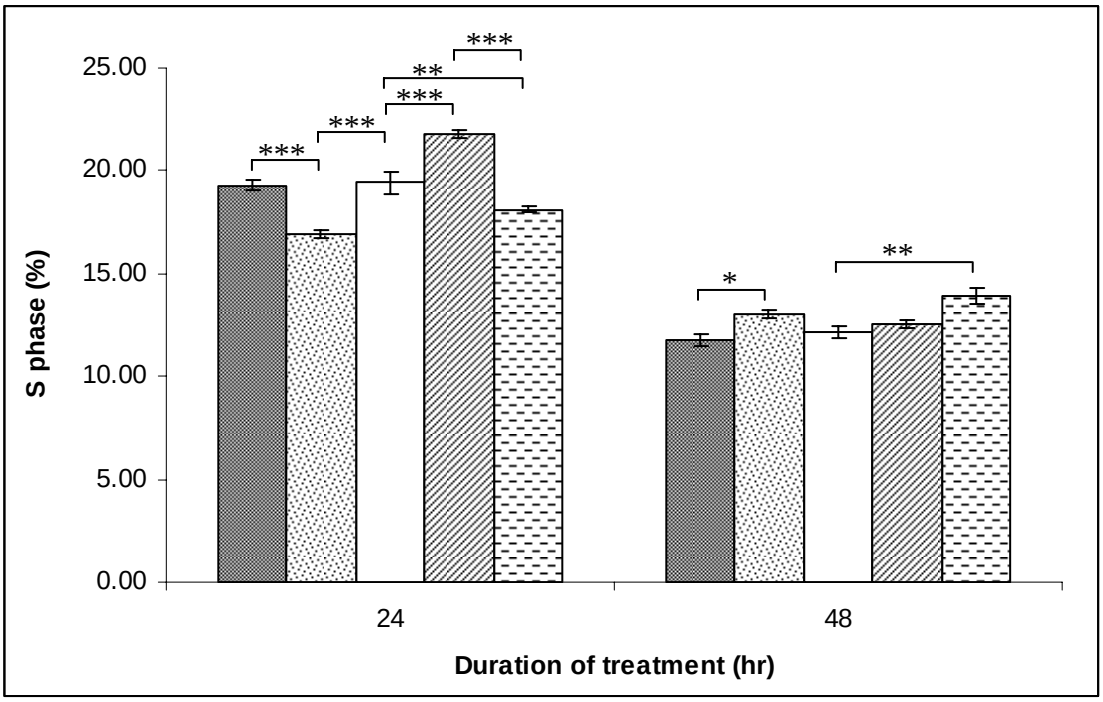

Figure 6.7: A comparison of the proportion of S phase Caco-2 cells following incubation with 5mM butyrate ▩, 5mM propionate ⁙, 5mM SCFA combination □ , 10mM butyrate ▨ and 10mM propionate ⁘. Data are presented as mean ± SEM (n = 6). *p < 0.05, **p < 0.01, ***p < 0.001.

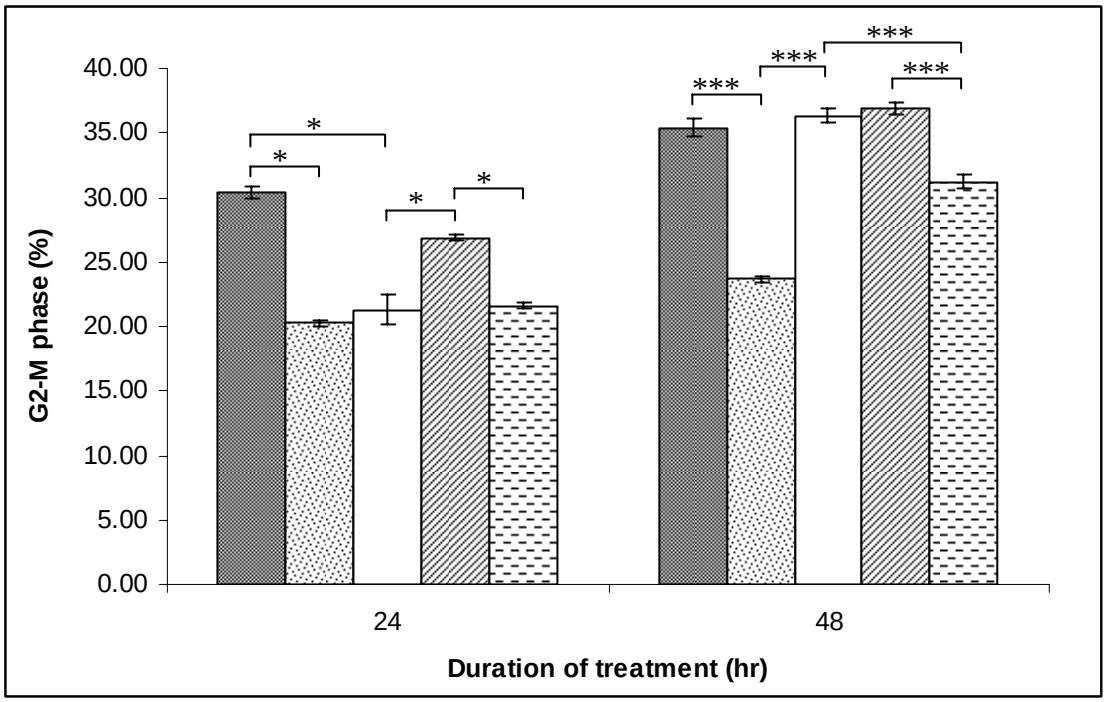

Figure 6.8: A comparison of the proportion of G2-M phase Caco-2 cells following incubation with 5mM butyrate ▩, 5mM propionate ⬚, 5mM SCFA combination□, 10mM butyrate ▨ and 10mM propionate ⬚. Data are presented as mean ± SEM (n = 6). *$p < 0.05$, ***$p < 0.001$.

6.4: Discussion

The present study described the effects of butyrate, propionate and a combination treatment containing both SCFAs, on cell viability and cell cycle regulation of the colon cancer cell line, Caco-2. All SCFA treatments reduced the viability of Caco-2 cells by inducing apoptosis and necrosis. Cell cycle regulation was altered by SCFA treatment leading to an increase in the percentage of Caco-2 cells within G2-M phase. Moreover, butyrate treatment alone, and in combination with propionate, induced a greater decrease in cell viability and a greater increase in the proportion of Caco-2 cells in G2-M phase than propionate alone.

Numerous investigations have reported the ability of butyrate to induce apoptosis in the Caco-2 colon cancer cell line [11, 37, 43, 79, 103]. Very few studies, however, have compared this with the effect of the SCFA, propionate, and even fewer have determined the effects of combined SCFAs. It was observed within the present study that butyrate alone, and the combination treatment containing both SCFAs, reduced the viability of Caco-2 cells and induced significantly greater apoptosis than propionate alone in this cell type. Indeed, further categorisation of apoptosis into early (EA) and late (LA) apoptosis indicated increases in both parameters by the SCFAs, although to a greater extent by butyrate and the combination SCFA treatment compared to propionate treatment alone. This study also noted a significant increase in the number of necrotic Caco-2 cells following SCFA treatment, however, butyrate and the combination SCFA treatment showed greater necrosis-inducing properties than propionate alone. These results indicate that butyrate and propionate are able to reduce the viability of Caco-2 cells through the induction of both pathways of cell death, apoptosis and necrosis. This is a unique finding, as previous studies have only reported the ability of SCFAs to induce apoptosis alone in Caco-2 cells while very few have described their necrosis-inducing effects. It is also interesting to note

the lack of any significant additive effect on the induction of apoptosis when the two SCFAs were combined. It is proposed that the combination SCFA treatment may represent a more physiologically relevant model when investigating the luminal effects of SCFAs *in vivo* than either agent alone.

The current study also assessed SCFA-induced alterations to cell cycle regulation in the Caco-2 colon cancer cell line. Previous studies have documented arrests of carcinoma cell lines in G1-phase [39, 43, 106] while others have reported G2-M phase arrests [82] following incubation with butyrate. A review of this literature highlighted three potential reasons for the lack of agreement between studies. Firstly, some studies have shown that the effects of butyrate are concentration-dependent whereby low butyrate concentrations (1-8mM) induced G0-G1 arrest while higher butyrate concentrations (8-16mM) induced G2-M phase arrest [83]. Secondly, Mariadason *et al*. [43] reported that the differentiation status of the colon cancer cell line dictated its response to butyrate. These investigators reported that Caco-2 cells became increasingly resistant to the effects of butyrate as they became more differentiated. Thirdly, there were differences in the methodologies between investigators, whereby some studies measured cell cycle in cell suspensions containing both viable and non-viable cells, while others used only viable (attached) cells.

The current study measured cell cycle alterations in total Caco-2 cell suspensions containing both viable and non-viable cells. It also followed the same pre-treatment protocol as Jones *et al*. [11] which allowed Caco-2 cells to incubate for 72hr prior to SCFA treatment. This enabled complete monolayer formation and allowed the assessment of SCFA-mediated effects on non-differentiated Caco-2 cells.

Butyrate, at all concentrations investigated, induced significant dose-dependent decreases in the proportion of Caco-2 cells undergoing G0-G1 phase while initially (≤ 24hr) increasing the number of cells in S-phase. Subsequently, after 48hr incubation with butyrate, a significant number of Caco-2 cells appeared to have moved through S-phase and were arrested in G2-M phase, confirming the observations of Archer *et al*. [82]. This result correlates with the greater extent to which cell death was induced within this cell line after 48hr than after 24hr incubation with butyrate. Additionally, no differences in cell cycle alterations were observed between treatment of Caco-2 cells with butyrate alone or in combination with propionate. Furthermore, propionate was noted to follow the same pattern of cell cycle alterations to that of butyrate, with decreases in G0-G1 phase and increases in S-phase and G2-M phase cells. However, the extent to which these changes were augmented by propionate was determined to be much less than following butyrate treatment, and primarily with only 5mM and 10mM propionate. In contrast, treatment with 1mM propionate induced an increase in the proportion of Caco-2 cells in G0-G1 phase and a significant decrease in G2-M phase cells after 48hr incubation. This author is unaware of any previous investigation that has reported this dose-dependent effect of propionate on Caco-2 cells. It is also of interest to note that propionate treatment alone induced significantly less changes to cell cycle regulation than the SCFA combination treatment.

In conclusion, butyrate, propionate and a combination of both SCFAs decreased cell viability and induced apoptosis and necrosis in the colon cancer cell line, Caco-2. Butyrate was documented to reduce Caco-2 cell viability to a greater extent than propionate alone or in combination. SCFA treatment was also shown to increase the proportion of Caco-2 cells in G2-M phase in contrast to many investigations by other laboratories. Therefore, the current study confirms the potential of SCFAs as adjunctive therapies in the treatment of colonic neoplasms.

CHAPTER 7: Short-chain fatty acids differentially alter intracellular protection and D-glucose metabolism in the Caco-2 colon carcinoma cell line

7.1: Introduction

Recent investigations within our laboratory have documented SCFA-mediated increases in oxidative pentose pathway (OPP) activity and reductions in glutathione (GSH) availability in the Kato III gastric cancer cell line (discussed in Chapter 4). Physiologically, gastric derived cell lines would not normally come into contact with SCFAs due to the almost complete metabolism of butyrate within the colon [33, 70, 107]. Therefore, the current study proposes that a colonic derived cell line, such as Caco-2, which would be embryonically programmed to utilise butyrate, may respond to SCFAs in a different manner. Indeed, a recent study reported increases in glutathione-S-transferase (GST) availability in normal colonocytes and HT29 colon adenocarcinoma cells following butyrate administration [48]. GST's are a family of enzymes that catalyse the conjugation of reduced GSH with many electrophilic compounds, such as reactive oxygen species (ROS), and hence enable their neutralisation to harmless metabolites. Pool-Zobel *et al*. [48] proposed that the up-regulation of GST production by butyrate in differentiated colonocytes would enhance toxicological defence mechanisms against oxidative stress that may, in turn, protect against carcinogenesis. However, increases in GST or GSH availability in colon cancer cells could lead to resistance to chemotherapeutic agents. In opposition, other investigators have shown a depletion of GSH availability by butyrate treatment in various cell lines, including Caco-2 (human colon cancer derived), MCF-7 (human breast cancer derived),

CC531 (rat colon cancer derived) and HT29 (human colon cancer derived) cells [49-51, 108]. Therefore, a lack of agreement exists regarding the effects of butyrate on intracellular protective mechanisms. Additionally, we are unaware of any related studies investigating the effects of propionate on GSH availability in cancer cell lines.

It is becoming increasingly evident that an understanding of cellular metabolism within various cell types, such as cancer cells, is extremely important. Alterations to intracellular redox state and gene expression, by particular agents, may induce metabolic changes, such as alterations to glucose metabolism and OPP activity. Boren *et al*. [38] reported an inhibition of glucose oxidation following butyrate treatment in HT29 cells. They concluded that the utilisation of glucose as a primary carbon source was replaced by butyrate in a dose-dependent manner in this cell type. Concomitantly, up-regulated expression of alkaline phosphatase, a marker of differentiation, was observed with increasing doses of butyrate. Butyrate is known to induce differentiation in many malignant cell lines and thus may act, at least in part, by forcing a metabolic phenotype similar to differentiated cells. Further assessment of metabolic profiles within cancer cells, such as Caco-2 cells, prior to and following, SCFA treatment, may provide an insight into how cells are able to sense and respond to changes in their nutrient environment.

In this chapter, the metabolic and redox-associated effects of SCFA treatment are determined in the Caco-2 colon carcinoma cell line. Specifically, it is the aim of this study to assess OPP activity, GSH availability and D-glucose metabolism in the Caco-2 cell line following incubation with butyrate, propionate and a combination of both SCFAs. This could provide a mechanistic understanding of the mode of action of SCFAs on colon cancer cells.

7.2: Materials and methods

7.2.1: Materials

Caco-2 colon cancer cells were obtained from American Type Culture Collection (ATCC; Manassas, VA, USA; ATCC#: HTB-37). Dulbecco's modified Eagle's medium (DMEM), heat inactivated foetal bovine serum (FBS), Penicillin/Streptomycin (10,000units/ml, 10,000µg/ml), Dulbecco's phosphate buffered saline (DPBS) and Trypsin-EDTA were supplied by Invitrogen (Gibco®, Invitrogen Australia, VIC, Australia). Sodium butyrate, sodium propionate, potassium phosphate, $NADP^+$, EDTA disodium salt dihydrate, glucose-6-phosphate (G6P) disodium salt, 5,5'-dithiobis (2-nitrobenzoic acid; DTNB) and glucose-6-phosphate dehydrogenase (G6PDH) were obtained from Sigma-Aldrich (Castle Hill, NSW, Australia). GSH and GSH reductase were supplied by Roche Diagnostics (Castle Hill, NSW, Australia). 1-^{13}C-D-glucose was obtained from Sercon Australia (Fulham Gardens, SA, Australia). Stock (200mM) sodium butyrate, sodium propionate and an equal combination of both (1:1) were prepared using sterile water and frozen at -20°C prior to the experiments.

7.2.2: Caco-2 cell culture

Caco-2 cells were cultured in 20ml complete DMEM medium containing 10% FBS and Penicillin/Streptomycin (90U/ml; 90µg/ml) in 75cm^2 cell culture flasks. Cells were maintained in a humidified 5% CO_2 incubator at 37°C.

7.2.3: Experimental design

For the determination of G6PDH activity and D-glucose metabolism, Caco-2 cells were seeded into 75cm^2 cell culture flasks ($1.0x10^7$ viable cells/flask) in 20ml complete DMEM medium and incubated for 72hr at 37°C prior to treatment. Cell culture medium was replaced with 20ml fresh

DMEM after 48hr incubation. Stock sodium butyrate, sodium propionate (100µl, 500µl or 1000µl) or the combination treatment (500µl) were then added to culture flasks in triplicate to give final concentrations of 1mM, 5mM and 10mM SCFA. Control cells received sterile water (500µl). Cells were incubated at 37°C and harvested after 24hr and 48hr incubation (described below) and immediately assessed for G6PDH activity and D-glucose metabolism.

For the determination of GSH availability, Caco-2 cells were seeded into 6 well plates ($1.0x10^6$ viable cells/well) in 2ml complete DMEM medium and incubated for 72hr at 37°C prior to treatment. Medium was replaced with fresh DMEM after 48hr incubation. Stock sodium butyrate, sodium propionate (10µl, 50µl or 100µl) or the combination of both SCFAs (50µl) were then added to the wells in duplicate to provide final concentrations of 1mM, 5mM or 10mM. Control cells received sterile water (50µl). Cells were incubated at 37°C and harvested after 24hr and 48hr. All cells from duplicate wells were removed and centrifuged at 156 x g for 5min. Supernatants were removed and cells resuspended in DMEM (1ml) and immediately assessed for cell number by exclusion of 0.1% nigrosine before being frozen at -80°C until analysis.

7.2.4: Cell harvesting

Caco-2 cells were harvested using a similar method to that described in Chapter 6 (6.2.4). However, the assessment of G6PDH activity, GSH availability and D-glucose metabolism in the current study, were undertaken in viable cells only. All culture medium containing non-adherent cells was discarded.

7.2.5: Assays

7.2.5.1: G6PDH activity

G6PDH activity was assessed using the method described in Chapter 2 (2.2.5).

7.2.5.2: GSH availability

GSH availability was assessed using the method described in Chapter 4 (4.2.4.3). However, samples were not diluted in Triton X-100 lysing solution when added to the microplate within this study due to initial experiments that had indicated lower levels of GSH availability in Caco-2 than Kato III cells.

7.2.5.3: 1-^{13}C-glucose oxidation

D-glucose oxidation was assessed in Caco-2 cells following incubation with 5mM butyrate, propionate and SCFA combination mix as described previously in chapter 2 (2.2.4).

7.2.6: Statistics

Data are presented as mean ± SEM. Data from each treatment group were initially analysed using Two-way ANOVA's with Fisher's LSD *post-hoc* tests. In order to compare the effects of different SCFAs from separate experiments, G6PDH and D-glucose metabolism data were transformed to 'change from control' values. This was carried out by subtracting measured values for each treatment group from control values for each experiment. This was not undertaken for GSH experiments which were all carried out at the same time. It must be noted that only 5mM and 10mM concentrations of each SCFA treatment were compared in this setting. Statistical analyses comparing differences in the ability of butyrate, propionate and the combination SCFA treatment to alter each parameter were analysed at individual time points using Kruskal-Wallis One-way ANOVA on Ranks with Student-Newman-Keuls *post-hoc* tests.

All statistics were calculated using SigmaStat 3.0 (SYSTAT Software Inc., California, USA). Significance was assumed at $p < 0.05$.

7.3: Results

7.3.1: Oxidative pentose pathway activity

7.3.1.1: Butyrate increased G6PDH activity in Caco-2 cells

Incubation with butyrate increased G6PDH activity after 24hr (5mM, $p < 0.001$, $n = 9$; 10mM, $p < 0.001$, $n = 9$) and 48hr (10mM, $p < 0.05$, $n = 9$) compared to control values (Figure 7.1). G6PDH activity was significantly greater after 24hr incubation with 10mM and 5mM butyrate than 1mM butyrate ($p < 0.001$). After 48hr incubation, 1mM butyrate significantly reduced G6PDH activity ($p < 0.05$, $n = 9$) while no difference was observed with 5mM butyrate treatment ($p > 0.05$) compared to control. However, 10mM butyrate induced significantly greater G6PDH activity than the 5mM ($p < 0.01$) and 1mM ($p < 0.001$) concentration of butyrate after 48hr incubation.

7.3.1.2: Propionate increased G6PDH activity in Caco-2 cells

Propionate significantly increased G6PDH activity after 24hr (5mM, $p < 0.001$, $n = 9$; 10mM, $p < 0.001$, $n = 9$) and 48hr (1mM, $p < 0.001$, $n = 9$; 5mM, $p < 0.001$, $n = 9$; 10mM, $p < 0.001$, $n = 9$) compared to untreated cells (Figure 7.2). After 24hr, 5mM and 10mM butyrate induced greater increases in G6PDH activity than 1mM butyrate ($p < 0.001$). Interestingly, after 48hr incubation, 1mM butyrate induced G6PDH to a greater extent in Caco-2 cells than the 5mM and 10mM concentration of butyrate ($p < 0.001$).

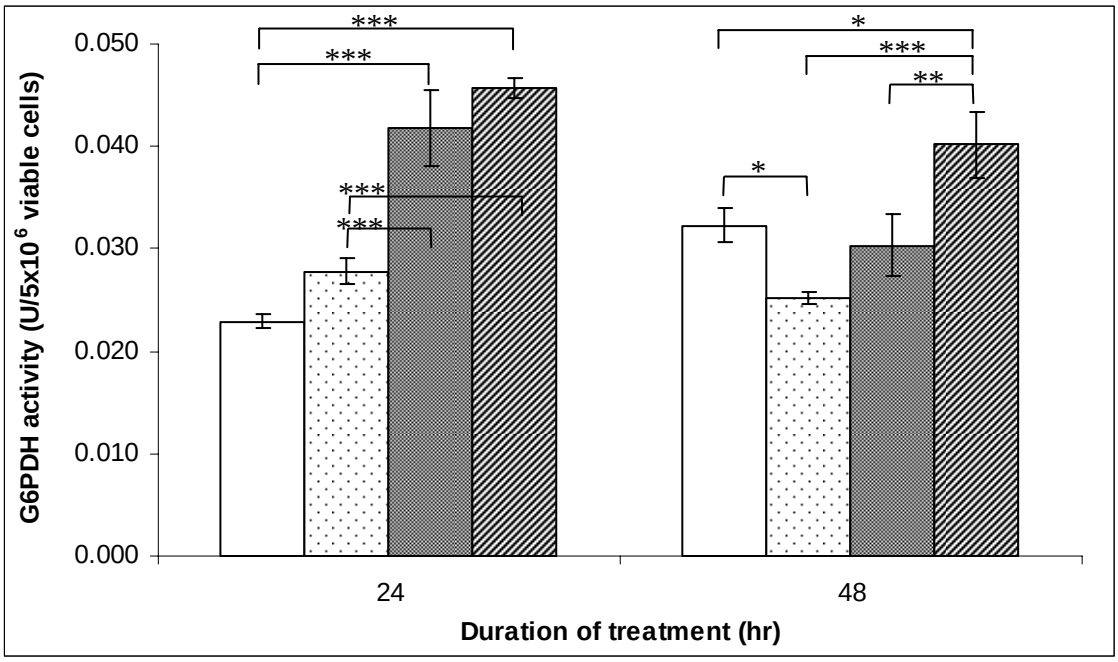

Figure 7.1: OPP activity in Caco-2 cells following incubation with 1mM ⬚, 5mM ▒ or 10mM ▨ butyrate compared to vehicle control ☐ . Data are presented as mean ± SEM (n = 9). *p < 0.05, **p < 0.01, ***p < 0.001.

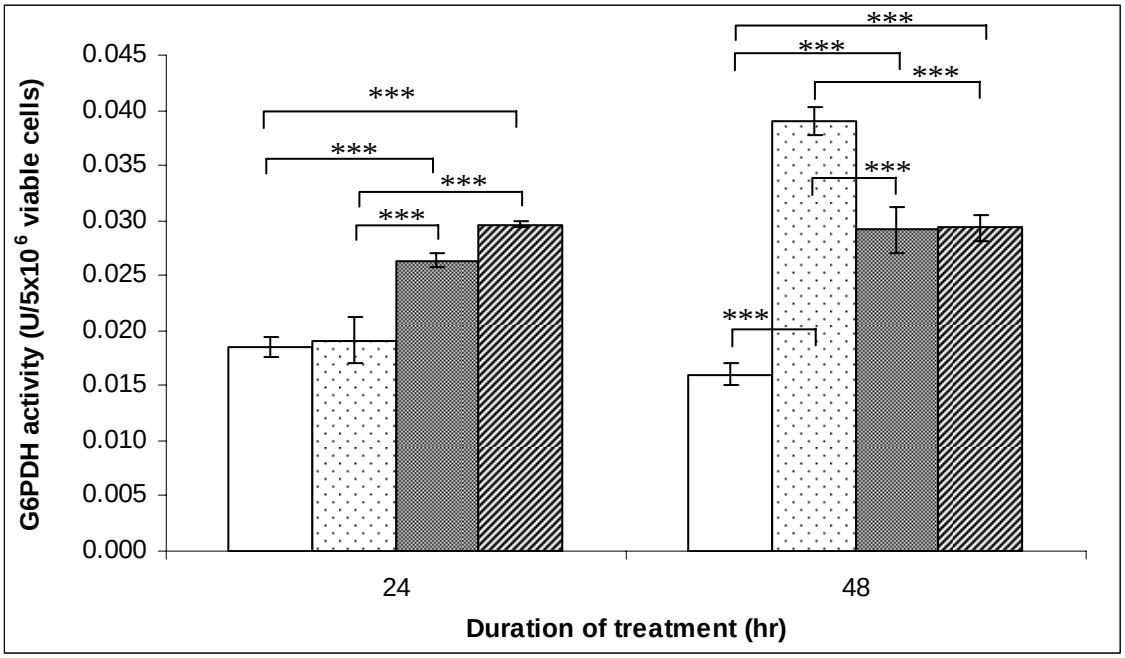

Figure 7.2: OPP activity in Caco-2 cells following incubation with 1mM , 5mM or 10mM propionate compared to vehicle control. Data are presented as mean ± SEM (n = 9). ***$p < 0.001$.

7.3.1.3: SCFA combination treatment increased G6PDH activity in Caco-2 cells

The 5mM SCFA combination treatment significantly increased G6PDH activity in Caco-2 cells after 24hr ($p < 0.001$, $n = 9$) and 48hr ($p < 0.05$, $n = 9$) compared to vehicle control values (Figure 7.3).

7.3.2: Comparison of OPP activity between SCFA treatment groups

7.3.2.1: Differences in SCFA-induced changes to G6PDH activity were time and dose-dependent.

After 24hr, no difference in G6PDH activity was observed in Caco-2 cells treated with 1mM butyrate or 1mM propionate ($p > 0.05$). However, 5mM and 10mM butyrate induced significantly greater increases in G6PDH activity than 5mM and 10mM propionate, respectively ($p < 0.05$). Incubation with the 5mM SCFA combination treatment for 48hr induced significantly greater G6PDH activities in Caco-2 cells than 5mM propionate ($p < 0.05$) but not 5mM butyrate ($p > 0.05$). All concentrations of propionate (1mM, 5mM and 10mM) induced significantly greater increases in G6PDH activity after 48hr incubation with equivalent concentrations of butyrate ($p < 0.05$). The 5mM SCFA combination treatment induced significantly greater increases in G6PDH activity than 5mM butyrate ($p < 0.05$). In contrast, 5mM SCFA combination treatment induced an increase in G6PDH activity that was significantly less than 5mM propionate ($p < 0.05$).

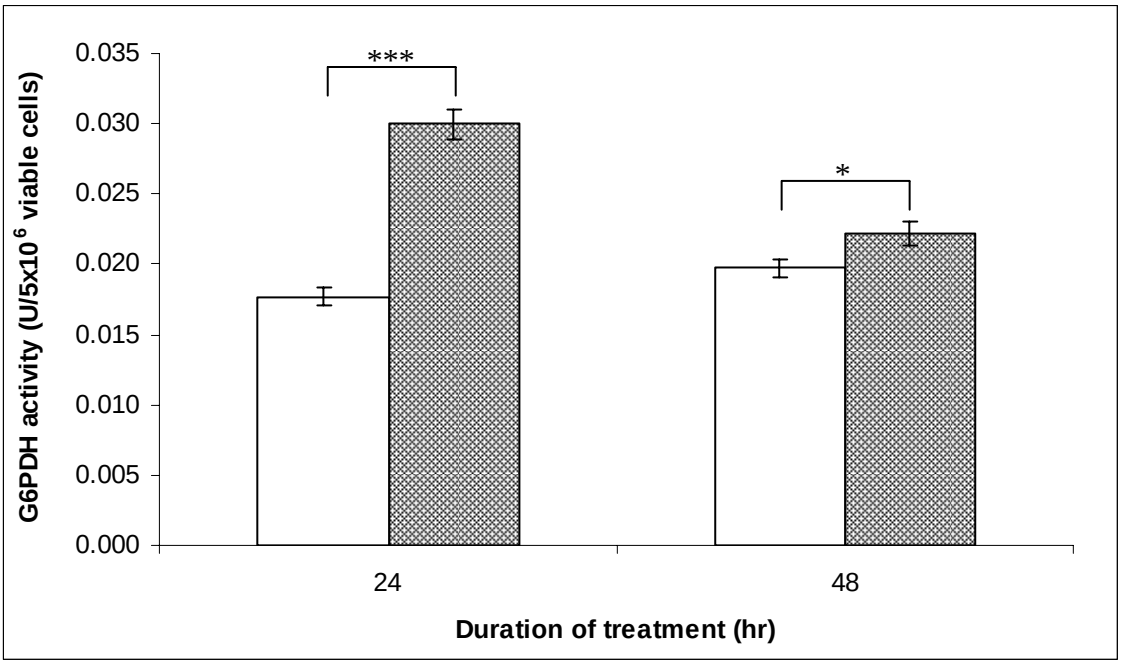

Figure 7.3: OPP activity in Caco-2 cells following incubation with 5mM SCFA combination treatment ▨ compared to vehicle control □ . Data are presented as mean ± SEM (n = . *p < 0.05, ***p < 0.001.

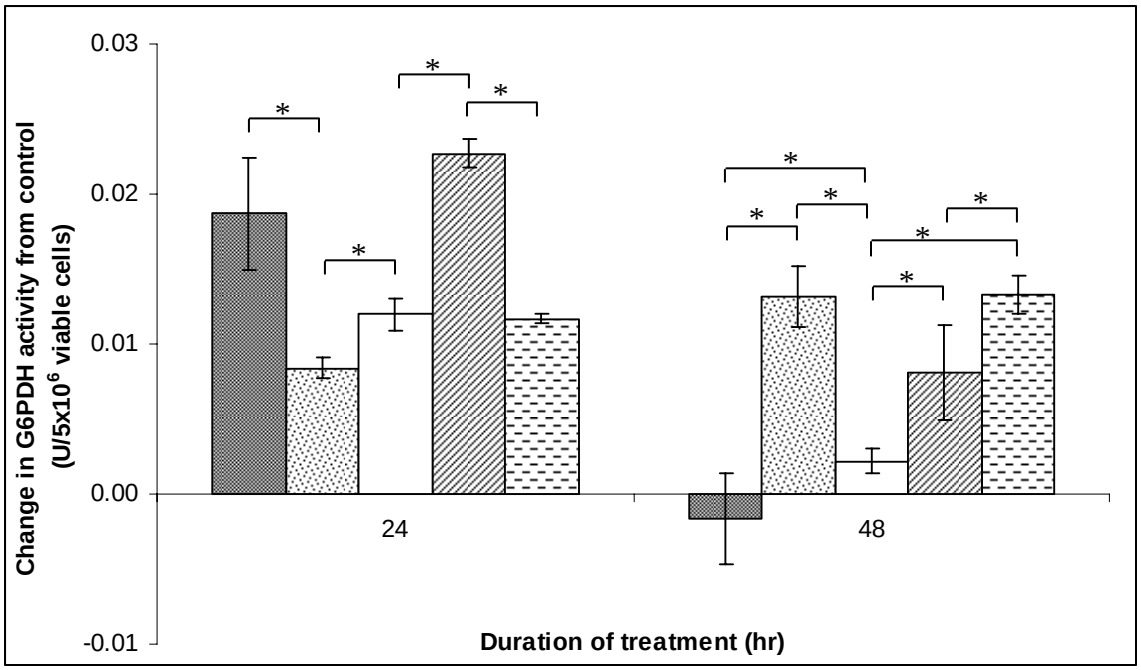

Figure 7.4: A comparison of OPP activities in Caco-2 cells following incubation with 5mM butyrate ▩, 5mM propionate ⁙, 5mM SCFA combination □, 10mM butyrate ▨ and 10mM propionate ⁘. Data are expressed as 'change in G6PDH activity from control values' and presented as mean ± SEM (n = 9). *$p < 0.05$.

7.3.3: GSH availability

7.3.3.1: Butyrate reduced GSH availability in Caco-2 cells

Butyrate reduced GSH availability in Caco-2 cells following 24hr (5mM, $p < 0.001$, $n = 8$); 10mM, $p < 0.001$, $n = 8$) and 48hr (5mM, $p < 0.001$; $n = 8$; 10mM, $p < 0.001$, $n = 8$) compared to control values (Figure 7.5). Incubation with 5mM and 10mM butyrate reduced GSH availability to a greater extent than 1mM butyrate after 24hr ($p < 0.001$). After 48hr incubation, GSH availability was reduced to a greater extent after 5mM ($p < 0.001$) and 10mM ($p < 0.01$) butyrate than 1mM butyrate.

7.3.3.2: Propionate reduced GSH availability in Caco-2 cells

Incubation with propionate significantly reduced GSH availability after 24hr (1mM, $p < 0.001$, $n = 8$; 5mM, $p < 0.001$, $n = 8$; 10mM, $p < 0.001$, $n = 8$) and 48hr (1mM, $p < 0.001$, $n = 8$; 5mM, $p < 0.05$, $n = 8$; 10mM, $p < 0.001$, $n = 8$) compared to control (Figure 7.6). No differences in the reduction of GSH activity were observed after 24hr incubation with any concentration of propionate. However, 48hr incubation with 10mM propionate reduced GSH availability to a greater extent than 5mM propionate ($p < 0.01$) but not 1mM propionate ($p > 0.05$).

7.3.3.3: SCFA combination treatment reduced GSH availability in Caco-2 cells

Incubation with 5mM SCFA combination treatment reduced GSH availability in Caco-2 cells after 24hr ($p < 0.001$, $n = 8$) and 48hr ($p < 0.01$, $n = 8$) compared to vehicle control values (Figure 7.7).

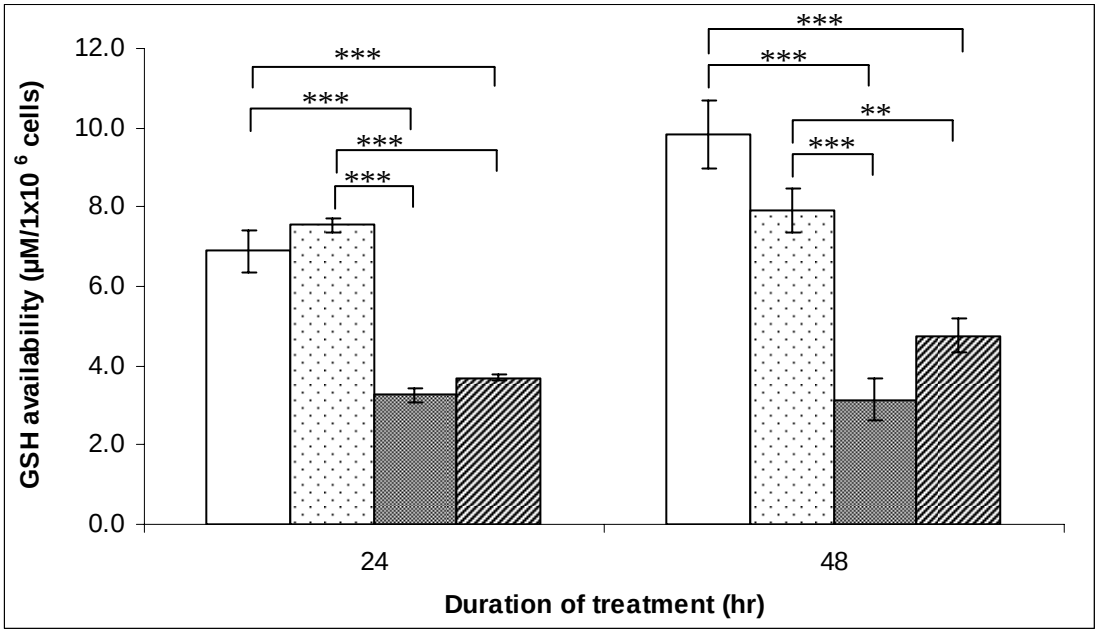

Figure 7.5: GSH availability in Caco-2 cells following incubation with 1mM ⬚, 5mM ▒ or 10mM ▨ butyrate compared to vehicle control □. Data are presented as mean ± SEM (n= 8). **p < 0.01, ***p < 0.001.

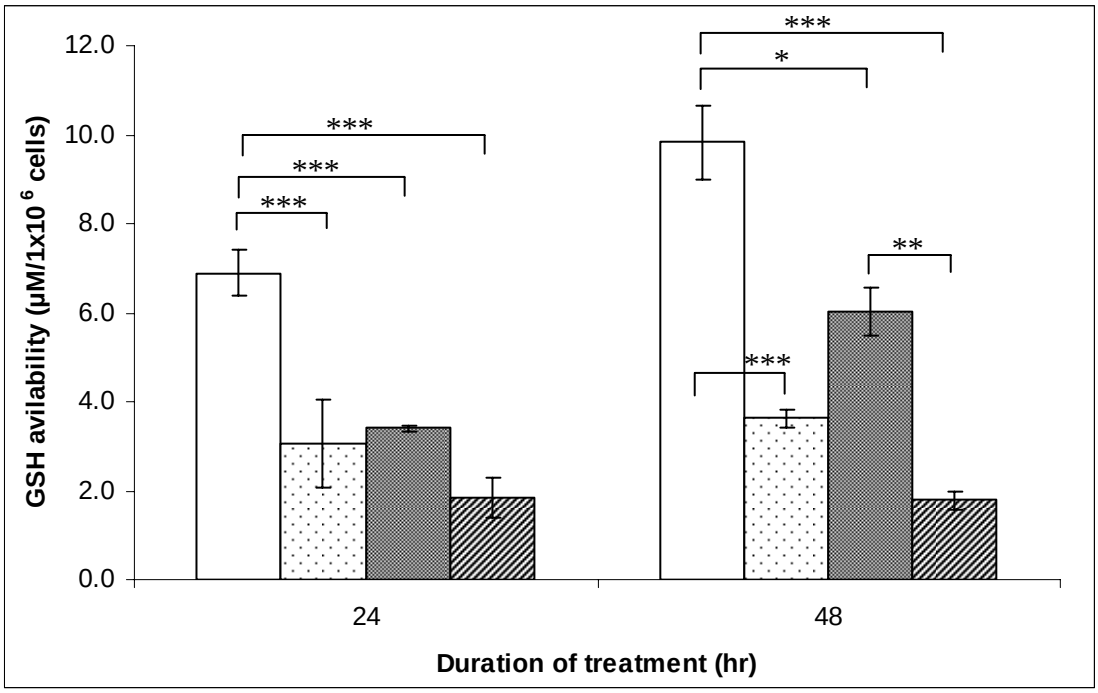

Figure 7.6: GSH availability in Caco-2 cells following incubation with 1mM 5mM or 10mM propionate compared to vehicle control. Data are presented as mean ± SEM (n = 8). **p < 0.01, ***p < 0.001.

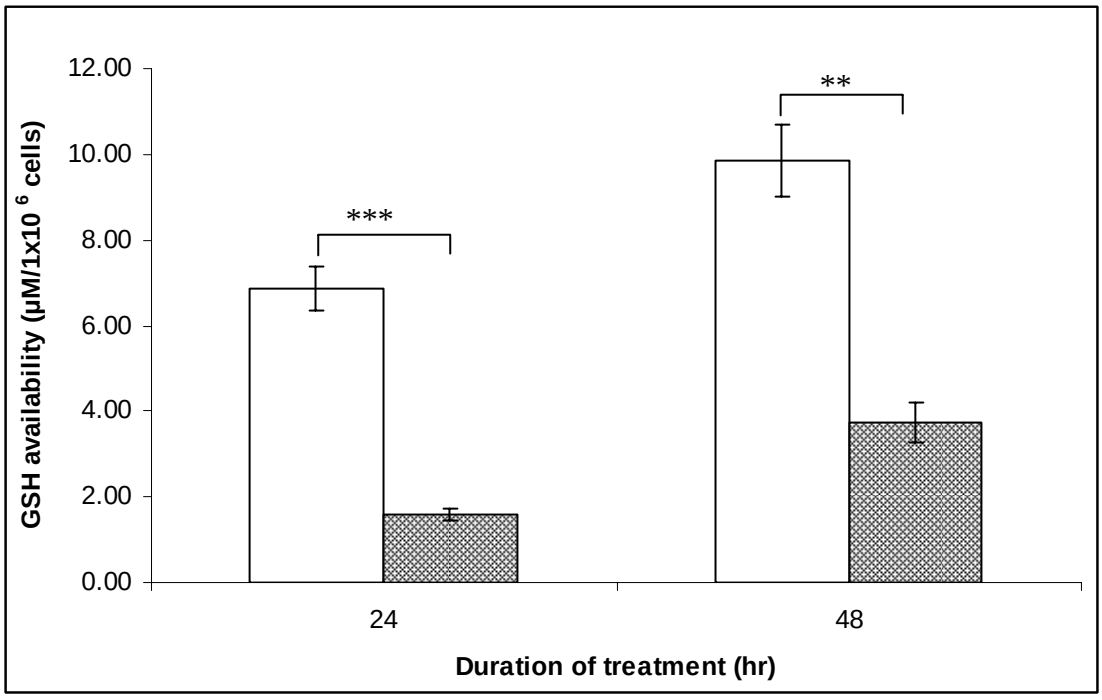

Figure 7.7: GSH availability in Caco-2 cells following incubation with 5mM SCFA combination treatment ▨ compared to vehicle control□ . Data are presented as mean ± SEM (n = 8). **p < 0.01, ***p < 0.001.

7.3.4: Comparison of GSH availability between SCFA treatment groups

7.3.4.1: SCFA treatments differentially reduced GSH availability in Caco-2 cells

After 24hr and 48hr incubation, 10mM propionate reduced GSH availability to a significantly greater extent than 10mM butyrate, respectively ($p < 0.05$) (Figure 7.8). Incubation with 5mM SCFA combination treatment for 24hr reduced GSH availability greater than 5mM butyrate ($p < 0.05$), 5mM propionate ($p < 0.05$) and 10mM butyrate ($p < 0.05$). After 48hr incubation, 5mM butyrate induced a greater decrease in GSH availability in Caco-2 cells compared to 5mM propionate ($p < 0.05$) and 5mM SCFA combination treatment ($p < 0.05$). In contrast, incubation with 5mM SCFA combination treatment led to a greater reduction in GSH availability compared with 5mM propionate alone ($p < 0.05$).

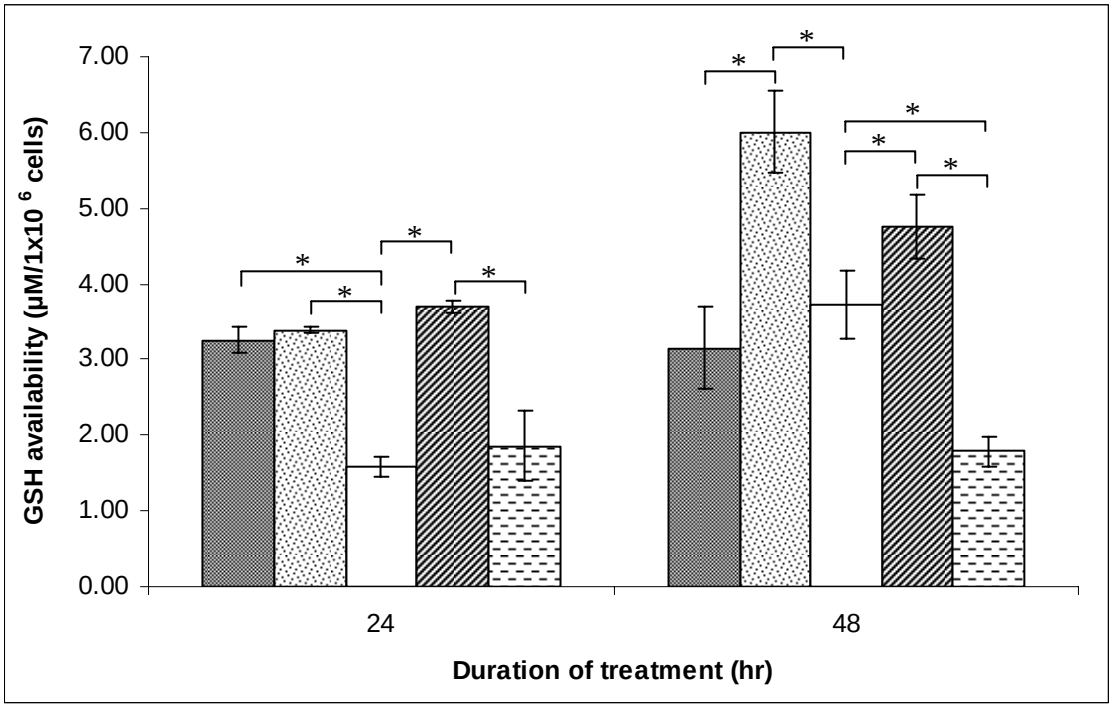

Figure 7.8: A comparison of GSH availability in Caco-2 cells following incubation with 5mM butyrate ▩ , 5mM propionate ▨, 5mM SCFA combination □, 10mM butyrate ▨, and 10mM propionate ▨. Data are presented as mean ± SEM (n = 8). *$p < 0.05$.

7.3.5: D-glucose metabolism

7.3.5.1: Butyrate increased D-glucose metabolism in Caco-2 cells

Butyrate increased D-glucose oxidation after 24hr ($p < 0.001$, $n = 5$) and 48hr ($p < 0.001$) compared to vehicle control values (Figure 7.9).

7.3.5.2: Propionate increased D-glucose metabolism in Caco-2 cells

Incubation with propionate significantly increased D-glucose oxidation after 24hr ($p < 0.001$, $n = 5$) and 48hr ($p < 0.001$, $n = 5$) compared to vehicle control values (Figure 7.10).

7.3.5.3: SCFA combination treatment increased D-glucose metabolism in Caco-2 cells

Incubation with 5mM SCFA combination treatment significantly increased D-glucose oxidation in Caco-2 cells after 24hr ($p < 0.001$, $n = 5$) and 48hr ($p < 0.001$, $n = 5$) compared to vehicle control values (Figure 7.11).

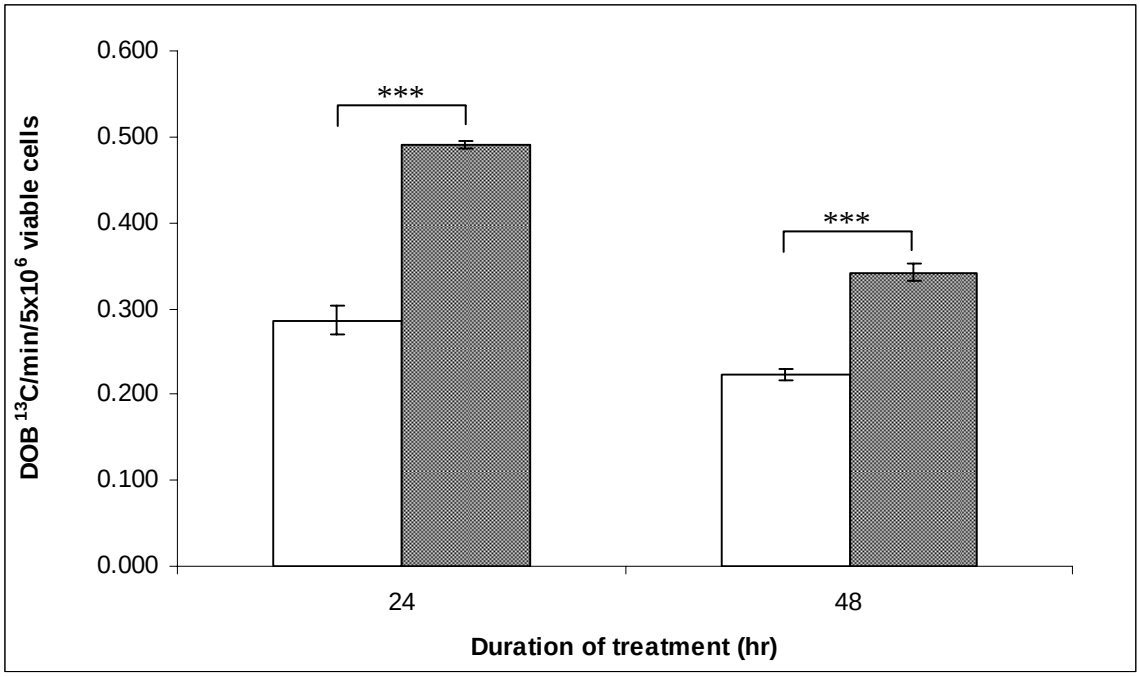

Figure 7.9: 1-^{13}C-D-glucose oxidation in Caco-2 cells following incubation with 5mM butyrate ▒ compared to vehicle control □. Data are presented as mean ± SEM (n = 5). ***$p < 0.001$.

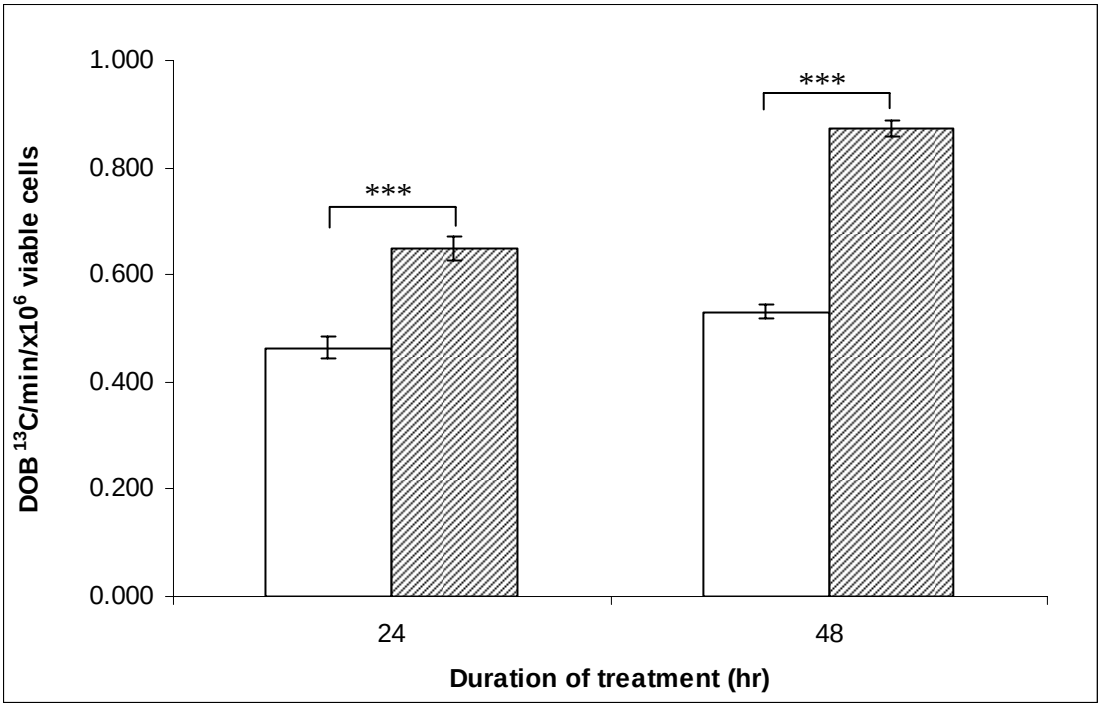

Figure 7.10: 1-^{13}C-D-glucose oxidation in Caco-2 cells following incubation with 5mM propionate ▨ compared to vehicle control □. Data are presented as mean ± SEM (n = 5). ***p < 0.001.

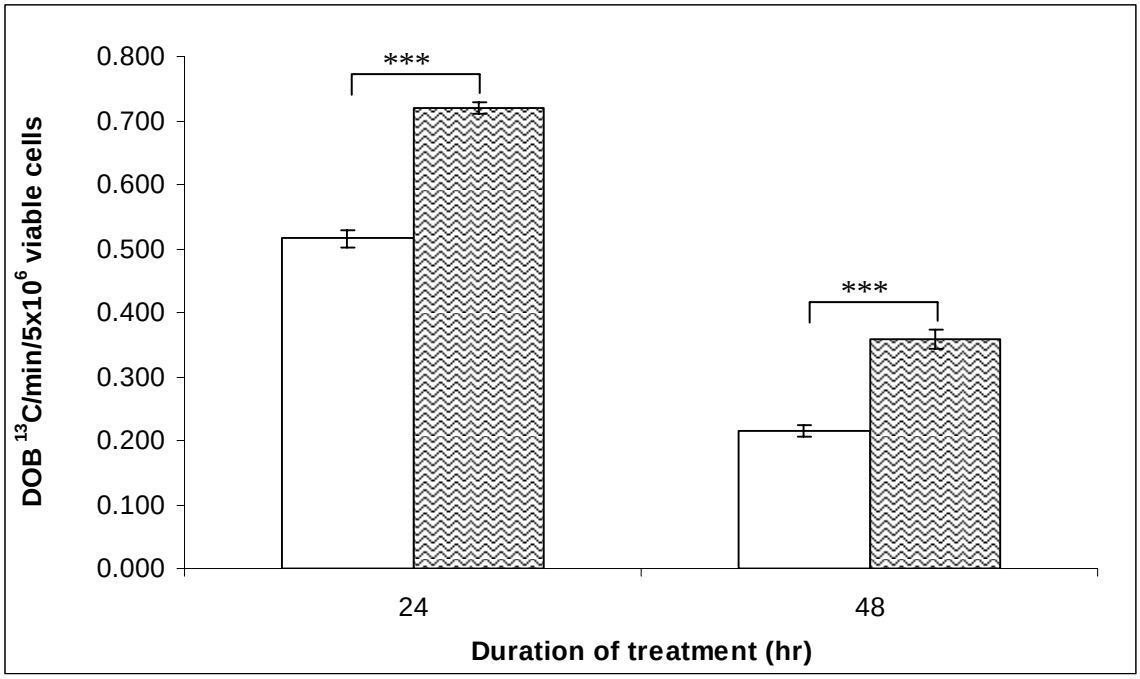

Figure 7.11: 1-^{13}C-D-glucose oxidation in Caco-2 cells following incubation with 5mM SCFA combination treatment ▨ compared to vehicle control □. Data are presented as mean ± SEM (n = 5). ***$p < 0.001$.

7.3.5: Comparison of D-glucose metabolism between SCFA treatment groups

7.3.5.1: Propionate increased D-glucose metabolism greater than butyrate or 5mM SCFA combination treatment

No statistically significant differences in 1-^{13}C-D-glucose oxidation were observed between any SCFA treatments after 24hr incubation when data were converted to 'change from control' values (Figure 7.12). However, after 48hr incubation, propionate significantly increased 1-^{13}C-D-glucose oxidation to a greater extent than 5mM butyrate ($p < 0.05$) and 5mM SCFA combination treatment ($p < 0.05$). No difference in 1-^{13}C-D-glucose oxidation was observed after 48hr incubation with 5mM butyrate and 5mM SCFA combination treatment ($p > 0.05$).

7.3.5.2: D-glucose metabolism increased time dependently with propionate but decreased with butyrate and combined SCFA treatment

1-^{13}C-D-glucose oxidation rates from 24hr SCFA treated Caco-2 cells were then compared to 48hr treated cells (Figure 7.12). Incubation with 5mM butyrate and 5mM SCFA combination treatment increased 1-^{13}C-D-glucose oxidation significantly less after 48hr than after 24hr ($p < 0.05$). In contrast, 48hr treatment with 5mM propionate increased 1-^{13}C-D-glucose oxidation greater than after 24hr ($p < 0.05$) incubation.

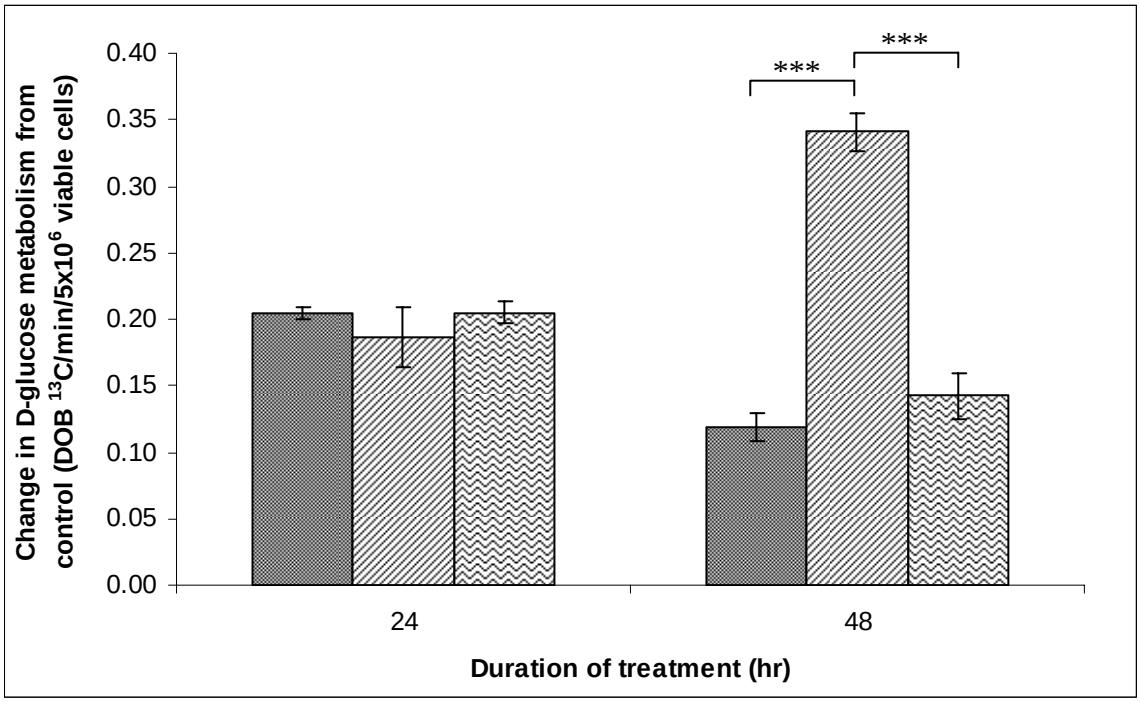

Figure 7.12: A comparison of the rates of D-glucose metabolism in Caco-2 cells following incubation with 5mM butyrate , 5mM propionate or 5mM SCFA combination treatments. Data are expressed as 'change in D-glucose metabolism from control levels' and presented as mean ± SEM (n = 5). ***$p < 0.001$.

7.4: Discussion

The current study investigated the effects of SCFA treatment on OPP activity, GSH availability and D-glucose metabolism in the Caco-2 colon carcinoma cell line. It was observed that OPP activity was increased while GSH availability was decreased following incubation with butyrate and propionate, alone or in combination. Furthermore, all SCFA treatments induced increases in D-glucose metabolism.

The OPP is an important pathway of glucose metabolism that plays a major role in regulating intracellular redox status in most cell types [21, 22, 109]. In agreement with the effects of SCFAs in Kato III cells in Chapter 4, the current study has demonstrated the differential and time-dependent modulation of OPP activity in Caco-2 cells by SCFAs. The current study observed that short-term incubation (24hr) with the higher concentrations of butyrate (5mM and 10mM) led to significantly greater increases in OPP activity than incubation with equivalent concentrations of propionate. Furthermore, the SCFA combination treatment (5mM) also induced greater increases in OPP activity than propionate alone (5mM) but not significantly different to the effects of butyrate alone (5mM). Interestingly, however, it was demonstrated that longer-term (48hr) treatment with propionate induced significantly greater increases in OPP activity than butyrate alone and SCFA combination treatment.

The observed increases in OPP activity by SCFAs are in agreement with recent work that reported butyrate-induced up-regulation of the OPP in HT29 colon cancer cells [38]. However, this is the first investigation to report propionate-induced increases in OPP activity in Caco-2 cells. An up-regulation in OPP activity indicates an increase in the oxidative state of the Caco-2 cell, probably by the production of ROS during treatment with SCFAs [50, 83-85]. Thus, a difference in the levels of OPP up-regulation, described within the current study, would suggest a

difference in the levels of ROS produced by each SCFA treatment. Therefore, these results indicate that in the short-term (24hr), butyrate treatment led to the production of significantly greater levels of ROS than either propionate or SCFA combination treatment. In contrast, as treatment duration continued (48hr), it suggests that ROS production was the greatest during propionate treatment. However, these results do not fit with the demonstrated greater induction of apoptosis by butyrate compared with propionate, described in Chapter 6.

The present study also demonstrated that both butyrate and propionate reduced GSH availability within Caco-2 colon cancer cells, although propionate caused the largest reductions. Indeed, the greatest reduction in GSH availability was obtained after incubation with 10mM propionate. However, short-term (24hr) incubation with the 5mM SCFA combination treatment reduced GSH availability to similar levels, but not after longer-term (48hr) incubation. Thus, the reductions in GSH availability by SCFA treatment observed within this study are in opposition to the increases in G6PDH activity described above.

Under normal conditions, in the absence of SCFAs, GSH levels would increase concomitantly with increases in OPP activity, to allow detoxification of ROS [21]. However, it appeared that the increase in OPP activity observed within the current study was unable to increase GSH availability to a degree that would outweigh the amount utilised during the detoxification process. Ultimately, this may have led to a reduction in the ability of Caco-2 cells to defend themselves against further ROS-induced oxidative stress. Again, however, a greater induction of ROS by propionate is in contrast to the enhanced level of apoptosis by butyrate in this cell type. Therefore, it is proposed that an induction of oxidative stress by SCFAs may not entirely explain their effect on apoptosis induction in this cell type, and this has been reported by recent studies

[48, 83, 85, 110]. Future studies aim to directly assess ROS production by SCFAs in this cell type.

Treatment of Caco-2 cells with SCFAs, within the present study, also induced differential and time-dependent changes in D-glucose metabolism. It was observed that all SCFA treatments induced similar increases in D-glucose metabolism after 24hr incubation. Propionate, however, increased D-glucose metabolism to a greater extent than butyrate alone or in combination with propionate after 48hr incubation. It is noteworthy that D-glucose metabolism also increased with propionate treatment from 24hr to 48hr. In contrast, decreases in D-glucose metabolism with butyrate alone and in combination with propionate occurred with increasing duration of treatment.

A previous study [38] reported that 72hr incubation of HT29 colon cancer cells with butyrate led to a decrease in D-glucose metabolism. The authors indicated that this was an adaptive change of HT29 cells to replace D-glucose with butyrate as the preferred energy source. Boren *et al*. [38] did not assess D-glucose metabolism prior to 72hr incubation. Therefore, we propose that the increased rate of D-glucose metabolism by butyrate within the present study, combined with the significant trend towards decreasing rates over time, reflect early changes (< 72hr) to D-glucose metabolism by Caco-2 cells. This may eventually (≥ 72hr) result in an inhibition of glucose metabolism and the preferential utilisation of butyrate, as documented by Boren *et al*. [38]. In opposition, propionate induced increases in the rates of D-glucose metabolism with increasing time. This suggests that propionate is not utilised as an energy source within Caco-2 cells, at least when D-glucose is present. Furthermore, increases in D-glucose metabolism by propionate may have reflected an increased movement of glucose through the OPP, highlighted by concomitant increases in G6PDH activity, discussed previously.

In conclusion, SCFA treatment induced differential and time-dependent alterations to OPP activity, GSH availability and D-glucose metabolism in the Caco-2 colon cancer cell line. The current study suggests that alterations to intracellular redox state and GSH availability only partially explain the effects of butyrate and propionate induced-changes to Caco-2 cell viability. It is proposed that differences in the metabolism of these SCFAs by this cell type may explain these disparities.

CHAPTER 8: A stable isotope-based profile of butyrate metabolism in Kato III and Caco-2 cell lines

8.1: Introduction

Butyrate is utilised primarily by colonic epithelial cells as a source of carbon for energy production [33, 38]. Previous studies have reported that a deficiency in the utilisation of butyrate by colonic epithelial cells may be involved in colon carcinogenesis [38, 70]. We hypothesised that differences in the ability of butyrate to induce apoptosis in Kato III and Caco-2 cell lines were due to differences in butyrate metabolism.

Recently, Boren *et al*. [38] investigated the metabolism of butyrate in the butyrate-sensitive colon adenocarcinoma cell line (HT29) and the butyrate-resistant pancreatic adenocarcinoma cell line (MIA) using stable isotope-based metabolic profiling. These investigators demonstrated that labelled butyrate was rapidly metabolised by HT29 (butyrate-sensitive) cells but poorly metabolised by MIA (butyrate-resistant) cells. Boren *et al*. [38] also reported a decrease in the metabolism of D-glucose by HT29 cells with increasing doses of butyrate. These investigators proposed that a change in metabolic phenotype of HT29 cells could have led to changes in the intracellular concentrations of many metabolites, such as glucose and fatty acids, that act as signals for transcriptional, translational and post-translational events.

In Chapters 3 and 6 of the current thesis it was demonstrated that butyrate induced apoptosis in the Kato III (gastric) and Caco-2 (colonic) cell lines. However, differences in the ability of butyrate to induce apoptosis were apparent between the two cell types. Indeed, a 6% increase in

the induction of apoptosis from control levels was observed after 48hr incubation with 5mM butyrate in Kato III cells compared to a 35% increase in apoptosis in Caco-2 cells. Thus, Caco-2 cells appeared to be more sensitive to butyrate than Kato III cells. It is proposed that this may have been due to an increased metabolism of butyrate by Caco-2 cells compared to Kato III cells.

The current study compared butyrate metabolism in the Kato III and Caco-2 cell lines using the novel CO_2 collection chamber developed within our laboratory (described in Chapter 2) and the stable isotope tracer, 1-^{13}C-butyrate.

8.2: Materials and methods

8.2.1: Materials

Kato III (HTB-103) and Caco-2 (HTB-37) cells were obtained from ATCC (Manassas, VA, USA). RPMI 1640, glucose-free RPMI 1640, DMEM, glucose-free DMEM, heat inactivated FBS, Glutamax, Hepes (1M) and Penicillin/Streptomycin (10,000units/ml, 10,000µg/ml) were supplied by Invitrogen (Gibco®, Invitrogen Australia, VIC, Australia). 1-^{13}C-butyrate (sodium salt) was obtained from Cambridge Isotope Laboratories Inc (MA, USA). A solution of 1-^{13}C-butyrate (10mM) was prepared by dilution in glucose-free RPMI or DMEM.

8.2.2: Cell culture

Kato III cells were cultured in 20ml complete RPMI 1640 medium containing 10% FBS, 4mM Glutamax, 20mM Hepes and Penicillin/Streptomycin (73.5U/ml; 73.5µg/ml) in 75cm^2 cell culture flasks. Caco-2 cells were cultured in 20ml complete DMEM medium containing 10% FBS and Penicillin/Streptomycin (90U/ml; 90µg/ml) in 75cm^2 cell culture flasks. Cells were maintained at 37°C in a humidified 5% CO_2 incubator.

8.2.3: Experimental design

For the assessment of butyrate metabolism in Kato III cells, cells were seeded into 75cm^2 cell culture flasks (1.5x10^7 viable cells/flask) in 20ml RMPI in triplicate and incubated at 37°C for 24hr. Kato III cells were then removed from glucose-containing RPMI medium by centrifugation (156 x g for 8min at 20°C), pooled and resuspended to a final concentration of 5x10^6 viable cells/ml in glucose-free RPMI 1640 containing Glutamax (4mM), Hepes (20mM) and Penicillin/Streptomycin (73.5U/ml; 73.5µg/ml). Butyrate metabolism was assessed immediately as described below.

For the assessment of butyrate metabolism in Caco-2 cells, cells were seeded into 75cm^2 cell culture flasks (1.0x10^7 viable cells/flask) in triplicate in 20ml DMEM and incubated at 37°C for 72hr as described previously (7.2.3). Caco-2 cells were then harvested by trypsinisation as described previously (7.2.4), centrifuged (156 x g for 8min at 20°C) and pooled prior to the resuspension of viable cells to a final concentration of 5x10^6 viable cells/ml in glucose-free DMEM containing Penicillin/Streptomycin (90U/ml; 90µg/ml). Butyrate metabolism was assessed immediately.

8.2.4: Assay of butyrate metabolism

Butyrate metabolism was assessed in Kato III and Caco-2 cells using a similar method to the measurement of 1-^{13}C-D-glucose metabolism described in Chapter 2 (2.2.4). Kato III and Caco-2 cell suspensions (containing 5x10^6 viable cells/ml) were aliquoted (1ml) into gas collection chambers (Figure 2.2) containing glucose-free RPMI (1.5ml; n = 5) or DMEM (1.5ml; n = 5). The 1-^{13}C-butyrate solution (10mM; 2.5ml) was then added to each chamber to give a final butyrate concentration of 5mM. Chambers were immediately capped and a needle was pierced through the cap and connected to a two-way tap. The flasks were then partially evacuated and subsequently filled with 150ml of a 5% CO_2/air gas mix. A sample of flask air (10ml) was sampled and injected into an exetainer tube (Exetainer®, Labco, High Wycombe, England). A 5% CO_2/air mixture (10ml) was injected back into the flask to maintain a constant concentration of CO_2 within the flask (approximately 2%). Samples of flask air were collected every 15min for 2hr. Post-trial cell viability counts were then undertaken on all samples using 0.1% nigrosine to correct $^{13}CO_2$ production rates for differences in cell numbers. All flask air samples were analysed for change in $^{13}CO_2/^{12}CO_2$ ratio by isotope ratio mass spectrometry (IRMS;

ABCA20/20 Europa Scientific). $^{13}CO_2/^{12}CO_2$ ratios were expressed as δ $^{13}CO_2$ values (‰) relative to the PeeDee Belemnite Limestone standard and changes in the δ ^{13}C level compared with baseline were expressed as δ over baseline (DOB ^{13}C). 1-^{13}C-butyrate metabolism was calculated as rate of change in DOB ^{13}C/min/$5x10^6$ viable cells by fitting a trend line to graphed DOB ^{13}C values from 15min to 120min.

8.2.5: Statistics

Data are presented as mean ± SEM. Data were compared using a Student's t-test. All statistics were calculated using SigmaStat 3.0 (SYSTAT Software Inc., California, USA). Significance was assumed with $p < 0.05$.

8.3: Results

8.3.1: Butyrate metabolism

8.3.1.1: Differential profiles of butyrate metabolism in Kato III compared to Caco-2 cells

The production of $^{13}CO_2$ by Kato III cells was evident after 15min incubation with 5mM 1-^{13}C-butyrate (Figure 8.1). A linear $^{13}CO_2$ production rate was determined from 30min to 120min, thus this interval was utilised for determination of overall butyrate metabolic rate in this cell type. In contrast, Caco-2 cells did not show any significant $^{13}CO_2$ production until 45min following administration of 1-^{13}C-butyrate (Figure 8.2). $^{13}CO_2$ production appeared linear from 60min to 120min, thus this interval was utilised to determine the overall rate of butyrate metabolism in this cell type.

8.3.1.2: Caco-2 cells metabolised butyrate significantly faster than Kato III cells

Caco-2 cells metabolised 1-^{13}C-butyrate at a significantly greater rate than Kato III cells (0.071 ± 0.002 vs. 0.046 ± 0.003; $p < 0.05$, $n = 5$) (Figure 8.3).

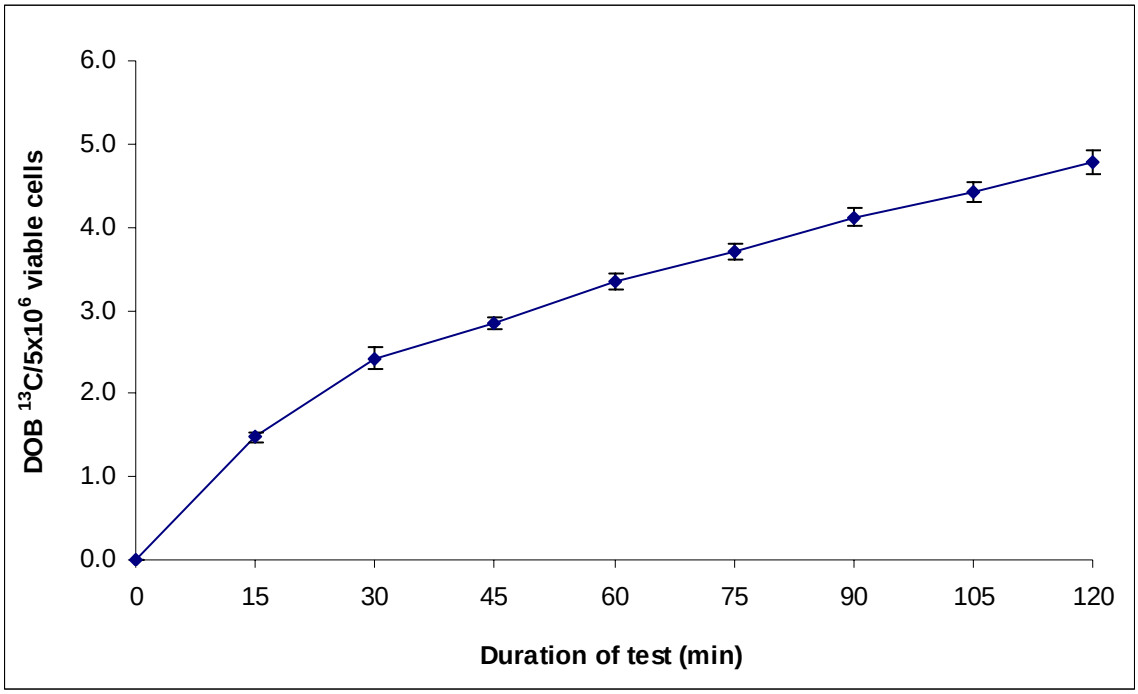

Figure 8.1: $^{13}CO_2$ production in Kato III cells during incubation with 1-^{13}C-butyrate. Note the linearity in $^{13}CO_2$ production from 30min to 120min. Data are presented as mean ± SEM (n = 6).

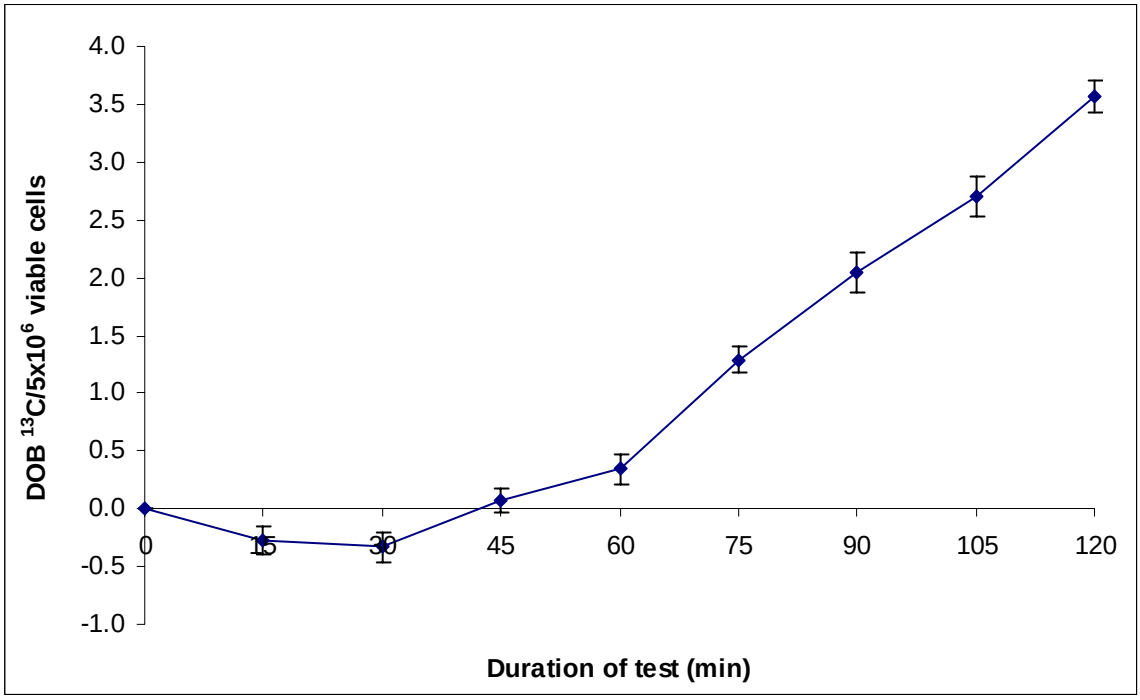

Figure 8.2: $^{13}CO_2$ production in Caco-2 cells during incubation with 5mM 1-^{13}C-butyrate. Note the linearity in $^{13}CO_2$ production from 60min to 120min. Data are presented as mean ± SEM (n = 6).

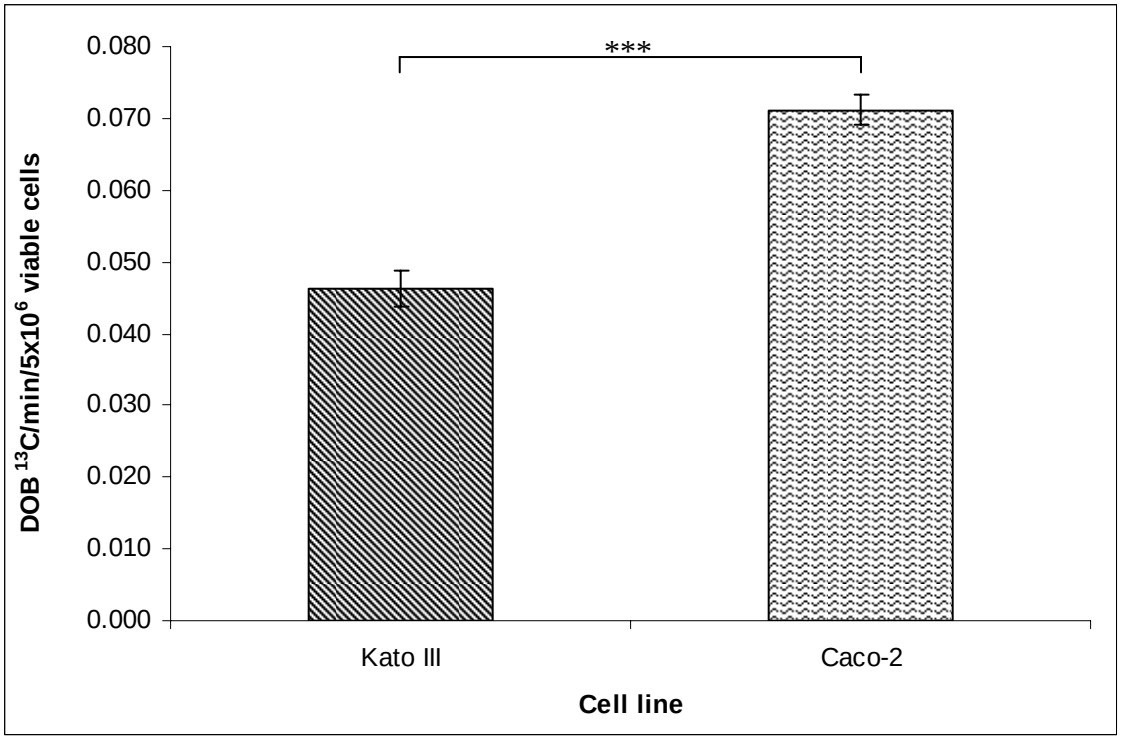

Figure 8.3: 1-^{13}C-butyrate metabolism in Kato III compared to Caco-2 cells. Data are presented as mean ± SEM. ***$p < 0.001$.

8.4: Discussion

Previous studies have proposed that the sensitivity of a cell to butyrate is dependent on its ability to metabolise this important SCFA [38, 70]. I have recently demonstrated that Kato III gastric cancer cells are less sensitive to butyrate-induced apoptosis than Caco-2 colon cancer cells. Therefore, the current study compared the rate of butyrate metabolism in the mildly butyrate-sensitive Kato III cell line and the highly butyrate-sensitive Caco-2 cell line. It was observed that Caco-2 cells metabolised butyrate at a significantly greater rate than Kato III cells.

Recent studies have reported the potential for butyrate as an adjunctive therapy to current chemotherapeutics in other cancer types, including leukaemia [110, 111] and gastric cancer [44, 87]. It is known, however, that differences exist in the sensitivity of various cancer cell lines to this SCFA [38, 112]. It has been proposed that these differences may be related to differences in the metabolism of butyrate [38]. Therefore, an appreciation of the metabolism of this SCFA in cell types other than colonocytes and transformed colonic cells is required if its mode of action is to be completely understood.

As discussed earlier, it has been proposed that a change from glucose to butyrate-derived energy production could significantly affect intracellular concentrations of many important metabolic products derived from glucose and fatty acids. These would be replaced with the metabolic intermediates of butyrate. In turn, this could severely affect the transcription and translation of genes involved in cell growth or apoptosis. This is in agreement with reported changes to the expression of many genes, including ornithine decarboxylase (differentiation marker) and p21 (cell cycle inhibitor) by butyrate [71, 79, 86].

This thesis has previously demonstrated that butyrate induces apoptosis in Caco-2 cells to a significantly greater extent than in Kato III cells. Additionally, the present study has documented a significantly greater rate of butyrate metabolism in Caco-2 cells than in Kato III cells. This is in parallel with greater rates of butyrate metabolism reported in butyrate-sensitive HT29 cells than butyrate-resistant MIA cells [38]. Therefore, it appears that the rate of butyrate metabolism is directly related to its ability to induce apoptosis.

In addition to differences in the overall rates of butyrate metabolism between the cell lines, the present study has also documented differences in the profile of butyrate metabolism. It was observed that Kato III cells showed an immediate (≤15min) increase in $^{13}CO_2$ production following the addition of 1-^{13}C-butyrate whereas Caco-2 cells appeared to take approximately 45min to induce any significant production of $^{13}CO_2$. Furthermore, in Kato III cells, the increase in $^{13}CO_2$ production was linear after 30min incubation while linearity was observed only after 60min incubation in Caco-2 cells.

Both cell lines had been removed from complete medium for the same period of time prior to the addition of 1-^{13}C-butyrate and cell viabilities were very similar prior to the experiments. Interestingly, this pattern of $^{13}CO_2$ production was not observed when Caco-2 cells were incubated with 1-^{13}C-D-glucose as discussed in Chapter 7. This suggests that the lag phase prior to the increase in $^{13}CO_2$ production by butyrate-treated Caco-2 cells is a phenomenon of butyrate metabolism by this cell type. No previous studies have documented this profile of butyrate metabolism in Caco-2 cells, or any other cancer cell type. However, this is mainly due to the lack of any previously available technique allowing a timed profile of butyrate metabolism in cell culture, unlike the technique utilised within this study.

In Caco-2 cells, the major transporter of butyrate into the cell is reported to be the monocarboxylate transporter 1 (MCT1) [107] although other mechanisms are thought to exist, such as an SCFA/HCO_3^- antiporter [113]. It is unknown whether Kato III cells also contain the MCT1 transporter and we are the first to report the metabolic rate of butyrate by this cancer cell type. It is proposed, however, that a gastric cancer cell line would not contain butyrate-specific transporters due to the lack of sufficient amounts of butyrate normally reaching the gastric epithelium. However, the Kato III cell line may contain a non-specific transporter that has the ability to co-transport butyrate. Recently, Daly and Shirazi-Beechey [79] reported the down-regulation of MCT1 expression in colon cancer cells. This is likely to reduce the transport of butyrate across the colonic cancer cell membrane. Potentially, this would result in the pattern of butyrate metabolism observed within this study.

In conclusion, the current study has documented differences in the profile and overall metabolism of butyrate in Kato III gastric cancer cells compared to Caco-2 colon adenocarcinoma cells. We propose that this is a major factor regulating the greater sensitivity of Caco-2 cells to butyrate-induced apoptosis than Kato III cells. Further studies are required to assess differences in butyrate metabolic pathways and gene expression in Caco-2 and Kato III cells that ultimately determine the action of this SCFA in cancer cells.

CHAPTER 9: Differential gene expression patterns by butyrate treatment in AGS gastric cancer cells and Caco-2 colon carcinoma cells: A pilot study.

The following chapter describes a pilot study undertaken during a three month study period spent at the Institute of Cell and Molecular Science, Centre for Gastroenterology, Whitechapel, UK. These studies were made possible by the following travel grants awarded to myself in 2005:

Gastroenterological Society of Australia: Astra-Zeneca Grant-In-Aid Award

Cancer Council SA Travel Grant

University of Adelaide: Department of Health Sciences Travel Award

University of Adelaide: Research Abroad Grant

University of Adelaide: Molecular and Biomedical Sciences Publication Awards

University of Adelaide: Molecular and Biomedical Sciences Travel Awards

9.1: Introduction

It is becoming increasingly apparent that the composition of nutrients within the luminal environment of the gut can have a direct effect on gene expression within the epithelium, and that this may influence cellular growth or death [79, 87, 114]. Considerable interest has been placed on the effects of butyrate in this setting. Indeed, a recent study in a human colon epithelial cell line demonstrated that butyrate induced the up-regulation of at least 59 genes, including $p21^{Waf1/Cip1}$, which is involved in cell cycle regulation, and the down-regulation of at least 162 genes, including Cyclin A2, which is involved in cell cycle progression [79]. Hence, the current study assessed the effects of butyrate on gene expression in the Caco-2 colon carcinoma cell line and in the AGS gastric cancer cell line.

Butyrate is a known histone deacetylase (HDAC) inhibitor, and many investigators have proposed that this mechanism may be responsible for a majority of its actions [41, 112, 115-117]. HDAC inhibitors are known to promote histone acetylation by relaxing DNA wrapped around core histones, thus allowing the transcription of particular genes [115]. Indeed, butyrate has been shown to induce hyperacetylation of histones leading to the up- or down-regulation of many genes, including Bcl-2 [41] and those described above.

A pilot study was conducted to determine the expression of a number of genes that may be regulated by butyrate. A brief summary of each gene assessed within this study is given herein:

9.1.1: Bcl-2 family of apoptosis-associated genes

The Bcl-2 family of mitochondrial membrane-bound proteins are known to be vitally important in the process of apoptosis [14, 118, 119]. These proteins include the anti-apoptotic Bcl-2 and

Bcl-X_L, which prevent apoptosis, and the pro-apoptotic Bax and Bak, which are potent triggers of apoptosis [14, 119-122]. Previous investigators have reported that over expression of Bcl-2 and Bcl-X_L prevented cells from undergoing apoptosis in response to various apoptosis-inducing agents, including H_2O_2 [14, 119]. Furthermore, Yang *et al*. [14] described that this effect was due to the prevention of cytochrome C release from the mitochondrial intermembrane space by Bcl-2 upregulation. In contrast, Naderi *et al*. [123] documented that the treatment of human diploid fibroblast (HDF) cells with H_2O_2 (oxidative stress) led to a significant increase in the availability of Bax protein within the mitochondrial membrane. Bax is known to permeabilise the mitochondrial membrane, facilitating the release of cytochrome C into the cytosol. In turn, cytochrome C is reported to bind to apoptosis-activating factor-1 (Apaf-1) and caspase-9 to produce an apoptosome, thus inducing the apoptotic pathway.

Recently, Choi *et al*. [124] measured the expression levels of Bcl-2, Bcl-X_L and Bax in human leukemic cells (U937) following treatment with butyrate. These investigators reported that butyrate induced a down-regulation of anti-apoptotic Bcl-2 and Bcl-X_L and the up-regulation of pro-apoptotic Bax expression. Therefore, the current study hypothesised that butyrate would induce changes to the expression of Bcl-2, Bcl-X_L and Bax that parallel those reported by Choi *et al*. [124] in both AGS and Caco-2 cells.

9.2.2: Ornithine decarboxylase (ODC)

Ornithine decarboxylase (ODC) is essential for cell growth and DNA synthesis through its role as a key enzyme in polyamine synthesis [15, 125]. In tumour cells, polyamines are reported to be both markers and regulators of neoplastic growth, and this has also been documented in the colon cancer cell line, Caco-2 [126]. Indeed, ODC is recognised as a cellular protooncogene and a vital factor contributing to oncogenesis. The activity of ODC is known to be increased in tumour cells

compared to non-transformed tissue and hence, ODC has long been considered a potential candidate in tumour prevention [126-128]. The effects of butyrate on ODC expression remain inconclusive.

9.2.3: $p21^{Waf1/Cip1}$

The progression of cells through the cell cycle is a tightly regulated process controlled by various cyclin-dependent kinases (CDKs) which are activated by cyclins and inhibited by CDK inhibitors [86]. The most widely studied CDK inhibitor is $p21^{Waf1/Cip1}$ and is responsible for G1-S phase arrest through activation of p53 by DNA damage. $p21^{Waf1/Cip1}$ has also been shown to be induced by p53-independent pathways during cellular differentiation [129].

Previous investigators have reported the capacity for butyrate to induce $p21^{Waf1/Cip1}$ in colon cancer cells which appears essential for butyrate-mediated cell cycle arrest [82, 86, 129, 130]. Importantly, Archer *et al*. [130] reported a butyrate-mediated induction of $p21^{Waf1/Cip1}$ induced, at least in part, by histone hyperacetylation. It was, therefore, proposed that butyrate was able to inhibit histone deacetylase at the level of the $p21^{Waf1/Cip1}$ gene allowing its transcriptional activation. However, we and others [82] have observed G2-M phase arrests in colon cancer cells (Caco-2) following butyrate treatment and this was also documented in Kato III gastric cancer cells within our laboratory. Hence, this study aimed to assess p21 gene expression in another gastric cancer cell type, AGS, compared to Caco-2 cells.

9.2.4: Caudal related homeobox-2 (Cdx-2)

Cdx-2 encodes an intestinal transcription factor of the homeoprotein family reported to regulate the development and homeostasis of the intestinal epithelium [131]. Many investigators have documented the capability of Cdx-2 to inhibit cell growth, stimulate cellular differentiation and promote apoptosis in a number of gastrointestinal cell lines [132-134]. Moreover, Cdx-2 has

been shown to be aberrantly expressed in gastric and colonic carcinomas and adenomas [133, 135] and is closely associated with expression of the intestinal mucin MUC2 gene [132]. Mesquita *et al*. [132] described enhanced expression of MUC2 in intestinal metaplasia and gastric carcinoma. Hence, previous investigators have proposed that Cdx-2 may play an important role in the aetiology of gastrointestinal carcinogenesis.

Domon-Dell *et al*. [131] investigated the expression of Cdx-2 in the human adenocarcinoma cell lines, Caco-2 and HT29, following butyrate treatment. It was observed that butyrate stimulated the expression of Cdx-2 in a dose and time-dependent manner. Thus, it appeared that the actions of butyrate may have been, in part, associated with its effects on the Cdx-2 gene which have not been previously reported. The effect of butyrate on Cdx-2 expression in gastric cancer cell lines is unknown.

9.2.5: Hairy and Enhancer of Split-1 (Hes-1)

Hes-1 is a transcriptional repressor gene of the Notch pathway that is important in determining the fate of intestinal epithelial stem cells during differentiation [136]. For example, Hes-1 appears to regulate whether intestinal epithelial stem cells adopt an exocrine/secretory (goblet cell/ enteroendocrine cell) fate or an absorptive (enterocyte) fate. The expression of Hes-1 is known to be regulated by Notch-1, another vitally important gene involved in the determination of cell fate [137, 138]. Recent investigations have documented that the constitutively active form of Notch-1 can act as an oncogene in leukaemia and breast cancer [137, 139]. In contrast, Notch-1 has been shown to act as a tumour suppressor gene in skin cancer [140].

The effects of butyrate on Notch signalling and Hes-1 expression are unknown. However, Stockhausen *et al*. [138] recently investigated the effects of the HDAC inhibitor, valproic acid, on Notch signalling, including Hes-1 expression, in a human neuroblastoma cell line. Valproic

acid has previously been shown to possess antitumorigenic activity and the ability to promote cellular differentiation in several tumour cell types [141, 142]. Valproic acid induced increases in Hes-1 expression and Notch signalling associated with cellular differentiation and increased cell death. Therefore, we hypothesised that butyrate could induce similar changes to Hes-1 expression to those revealed by valproic acid.

9.2.6: Musashi-1 (Msi-1)

The Msi-1 gene encodes an RNA binding protein required for the proliferation of undifferentiated progenitor cells, such as epithelial stem cells [143-147]. Msi-1 is reportedly expressed in the proliferative compartment of the colonic crypt while no expression is detected in the developing, differentiated compartment [147]. Hence, Msi-1 is thought to be a stem cell marker by many investigators [143, 144, 146, 147]. Recently, Battelli *et al*. [143] demonstrated that Msi-1 was able to alter the cell cycle profile through its regulation of $p21^{Waf\text{-}1/Cip}$. Specifically, overexpression of Msi-1 was observed to reduce $p21^{Waf\text{-}1/Cip}$ expression leading to a reduction in G1 phase and an increase in S and G2-M phases. Battelli *et al*. [143] also reported loss of Msi-1 expression was associated with significant increases in the expression of $p21^{Waf\text{-}1/Cip}$ inducing early exit from the cell cycle.

Msi-1 has also been described as a positive regulator of Notch signalling (discussed previously) through its suppression of the Notch antagonist, Numb [143, 146]. Moreover, Okano *et al*. [148] showed that Msi-1 induced activation of the Hes-1 gene (also discussed previously), a promoter of the Notch signalling pathway. Thus, Msi-1 has been proposed as a regulator of differentiation in intestinal epithelial cells. Many studies have indicated the capacity of butyrate to induce differentiation in colon cancer cells [82, 117, 149]. Hence, the current study hypothesised that butyrate would reduce Msi-1 expression in the Caco-2 cell line.

9.2.7: Summary

The present study investigated the effects of butyrate on expression of Bcl-2, Bcl-X_L, Bax, ODC, $p21^{Waf1/Cip1}$, Cdx-2, Hes-1 and Msi-1 in the AGS gastric adenocarcinoma cell line and the Caco-2 colon carcinoma cell, line using Real-Time PCR. The expression of Msi-1 was investigated in Caco-2 cells alone.

9.2: Materials and methods

9.2.1: Materials

AGS gastric cancer cells (ATCC#: CRL-1739) and Caco-2 colon carcinoma cells (ATCC#: 37) were obtained from American Type Culture Collection (ATCC; Manassas, VA, USA). High glucose Dulbecco's Modified Eagles Medium (DMEM), heat inactivated FBS, Hepes (1M), non-essential amino acids (NEAAs), Penicillin/Streptomycin (10,000units/ml, 10,000µg/ml) and TRIzol® Reagent were supplied by Invitrogen Life Technologies (Invitrogen UK, Inchinnan, UK). Sodium butyrate was obtained from Sigma-Aldrich (Poole, UK). Stock sodium butyrate was prepared using sterile water (200mM) and frozen at -20°C prior to the experiments. All reagents utilised for assessment of gene expression (primers, etc) were obtained from Promega (Madison, WI, USA) except for SYBR green which was obtained from QIAGEN (Crawley, West Sussex, UK).

9.2.2: Cell culture

AGS gastric adenocarcinoma cells were substituted for Kato III cells due to their ready availability within the laboratory in which the study was undertaken. For routine culture, AGS cells were cultured in high glucose DMEM (20ml) supplemented with 10% FBS and Penicillin/Streptomycin (90U/ml; 90µg/ml). Caco-2 colon carcinoma cells were cultured in high glucose DMEM supplemented with 10% FBS, NEAAs, Hepes (20mM) and Penicillin/Streptomycin (88U/ml; 88µg/ml). Cells were cultured in 75cm^2 flasks and incubated at 37°C in 5% CO_2 prior to experiments.

9.2.3: Experimental design

AGS and Caco-2 cells were seeded into 6 well plates in duplicate ($2x10^5$ cells/well) in 2ml of appropriate medium and incubated for 72hr prior to treatment with butyrate. Before treatment (-24hr), cell culture medium was replaced with a 5% FBS-containing medium which was otherwise identical to normal maintenance medium. This allowed synchronisation of cell cycle to G0-G1 phase [143]. Following this (24hr), stock sodium butyrate (10µl, 50µl and 100µl) was added to treatment wells to final concentrations of 1mM, 5mM and 10mM. Complete medium (50µl; 5% FBS) was added to control wells. Plates were incubated at 37°C and harvested after 24hr and 48hr as described below.

9.2.4: Cell harvesting

AGS and Caco-2 cells were harvested after incubation with butyrate using the same protocol as follows: All medium was carefully removed from wells using vacuum suction. TRIzol reagent, a ready to use reagent for the isolation of total RNA [150], was aliquoted (1ml) into each well and allowed to sit for 5min to allow for the complete dissolution of all cellular components. The cell-TRIzol suspension was then removed and placed in 1.5ml eppendorf tubes and frozen at -80°C until analysis.

9.2.5: RNA isolation from cell lines

Samples were thawed and allowed to sit at room temperature for at least 5min prior to RNA extraction. RNA was then extracted according to manufacturer's instructions (TRIzol Reagent protocol; Invitrogen, Catalogue #: 15596-026). Chloroform (200µl) was added to each sample followed by vortexing for 15sec and incubating at room temperature (RT) for 2 to 3min. Samples were then centrifuged (Sigma 3K20) at 12,000 x g for 15min at 4°C. Following centrifugation,

the sample separated into a red-phenol-chloroform phase (lower), an interphase (middle) and a colourless aqueous phase (upper). The aqueous phase, which exclusively contains the RNA, was transferred into a fresh eppendorf and the remainder was discarded. The RNA was precipitated from the aqueous phase by the addition of isopropyl alcohol (0.5ml) and an overnight incubation at 4°C. This was followed by centrifugation at 12,000 x g for 10min at 4°C causing the RNA precipitate to a gel-like pellet at the base of the tube. The supernatant was carefully removed and the RNA pellet washed once with 75% absolute ethanol (1ml). The sample was mixed by vortexing and centrifuged at 5,500 x g for 5min at 4°C. Supernatant was then removed and the pellet allowed to air dry briefly prior to redissolving in RNase-free water (20µl). The concentration of extracted total RNA was measured using a spectrophotometer (Heλios β, Unicam, Cambridge, UK) and the $A_{260/280}$ ratio of RNA was 1.0-1.6. Samples were stored at -20°C until further use.

9.2.6: Synthesis of cDNA from RNA

9.2.6.1: Reverse-Transcription Polymerase Chain Reaction (RT-PCR)

RNA samples (1µl: 0.5µg/µl) were added to 0.5ml thermo tubes (ABgene UK, Epsom, UK). To each thermo tube was then added a mix (11µl) containing Oligo dT primer (1µl), dNTP (10mM, 1µl, containing an equimolar mixture of dATP, dCTP, dGTP and dTTP) and RNase-free water (9µl). Thermo tubes were placed in a thermal cycler (MWG-Biotech Primus 96 plus, Milton Keynes, UK) and heated to 65°C for 5min before being placed on ice. A second mix (7µl), containing Moloney Murine Leukaemia Virus (M-MLV) buffer (4µl), RNasin (1µl) and RNase-free water (2µl), was added to each thermo tube. Tubes were then heated on a thermal cycler at 37°C for 2min. M-MLV reverse transcriptase was added (1µl) to each tube and incubated at

37°C for 50min followed by 15min incubation at 72°C. Thermo tubes were then returned to ice or stored at -20°C.

The integrity of each cDNA preparation was tested using gel electrophoresis by the detection of β-actin PCR products. β-actin is a housekeeping gene used as a marker of cDNA quality and gene expression (for example: [151]). Each cDNA sample (2μl) was added to a thermo tube containing a PCR mix (22.75μl). The PCR mix contained Mg-free buffer (x10; 2.5μl), dNTP mix (0.5μl), $MgCl_2$ (2μl), sense primer (0.3μl), antisense primer (0.3μl) and RNase-free water (17.15μl). Finally, Taq polymerase (0.25μl) was added to each thermo tube. Tubes were placed in the thermal cycler and the DNA was denatured at 95°C for 5min. DNA was then amplified by 30 cycles of 50sec at 58ºC (annealing temperature), followed by 50sec at 72ºC (polymerisation) and 50sec at 94ºC for denaturation. At the end of the amplification cycles, an additional polymerisation step was added for 7 minutes to "tidy-up" the incomplete ends of the product. Usually, 5μl of the PCR product is then run on an agarose gel to determine product formation. The rest can be stored at -20ºC.

9.2.7: Preparation of agarose gel

An agarose gel was prepared to run each DNA sample for detection of *β*-actin. Each agarose gel (2%) was prepared by the addition of agarose (4g; BDH Laboratory supplies, Poole, UK) to a TBE buffer (200ml). Stock TBE buffer (5X, 1L) contained Tris base (54g), boric acid (27.5ml) and EDTA (0.5M, 20ml) made up to 1L with water (pH 8.0). The agarose/TBE solution was heated to enable the agarose to dissolve and then allowed to cool to approximately 55°C prior to the addition of ethidium bromide (8μl; Sigma-Aldrich, Poole, UK). The molten agarose was poured into a gel frame and allowed to set before adding enough TBE buffer to cover the gel.

9.2.8: Gel electrophoresis

DNA samples were prepared for electrophoresis by the addition of a loading dye (5µl) containing bromophenol blue (0.25% w/v) and xylene cyanol FF (0.25% w/v) in a sucrose solution (40% w/v). Each DNA sample was carefully pipetted into the wells (15µl). A 100bp DNA ladder (100, 200, 300, 400, 500, 600, 700, 800, 900, 1000, 1500bp fragments) was added (6µl) at each end of the gel. The gel was run at 5V/cm until the bromophenol blue front had migrated a sufficient distance. Gels were then visualised using a UV trans-illuminator and then photographed.

9.2.9: Real-Time PCR

Real-Time PCR is an extraordinarily powerful technique that allows the quantitative analysis of gene expression. This technique utilises a DNA-binding dye (SYBR green) that incorporates into double stranded DNA (dsDNA) [152]. SYBR green is undetectable in its free form but emits fluorescence once bound to dsDNA, and the fluorescence signal increases proportionally with the increasing amount of amplicon generated with each cycle.

For each gene assessed using Real-Time PCR, a master mix was prepared containing SYBR green (7.5µl), custom primers (0.5µl, Table 3) and molecular grade water (5µl) (per sample). The master mix was aliquoted (13µl) into Real-Time PCR microtubes (Corbett Life Sciences, Sydney, NSW, Australia). Samples were then added to master mix-containing tubes (2µl) in duplicate and water (2µl) was added to tubes at the end of each primer set as control. To correct for experimental variation, each gene was normalised to the expression of the house-keeping gene, glyceraldehyde-3-phosphate dehydrogenase (GADPH) by the use of specific primers (Table 2) (Jones *et al*., 2005). Real-Time PCR was then performed and analysed on a Rotorgene

instrument (Rotorgene 3000, Corbett Life Sciences, Sydney, NSW, Australia) using the cycle settings described (Table 9.1).

After completion of the run, the threshold cycle (Ct) value (cycle at which a significant increase with an exponential growth of PCR product was first detected) was manually set (Personal communication, Dr James Wilson and Dr Meleri Jones). The Ct value is inversely proportional to the amount of product present. The threshold was placed above any baseline activity and within the exponential increase phase (which appears linear in the log transformation: Fig 1). The C_T of the gene of interest was normalised to the housekeeping gene (GAPDH) using the following algorithm: Relative expression (RE) = $2^{(\text{Ct GAPDH} - \text{Ct Gene of interest})}$. All gene expression levels are presented as RE from vehicle control values. Control values were standardised to a relative expression of RE = 1.00.

9.2.10: Statistics

Data are presented as mean ± SEM. The current study was undertaken as a pilot study to measure the expression levels of a broad range of genes. Therefore, no statistical analyses were performed on any of the data as each gene was analysed in duplicate only.

Step	Time	Temperature (°C)	Ramp
Initial activation	15min	95	20°C/sec
Denaturation	15sec	94	20°C/sec
Annealing	20-30sec	50-60	20°C/sec
Extension	10-30sec	72	20°C/sec

Table 9.1: Cycling conditions for Real-Time PCR.

Gene	Primer sequences (5’ to 3’)
GAPDH	Forward (For): ACAGTCCATGCCATCA
	TGCC
	Reverse (Rev): GCCTGCTTCACCACCT TCTTG
Bcl-2	For: GTGTGTGGAGAGCGTCAAC
	Rev: GAGACAGCCAGGAGAAATCA
Bcl-X_L	For: GTGCGTGGAAAGCGTAGAC
	Rev: GCTGCATTGTTCCCATAGAG
Bax	For: GCTGGACATTGGACTTCCT
	Rev: CTCAGCCCATCTTCTTCCA
ODC	For: AGGTTGGTTTCAGCATGTATC
	Rev: TCAGCTATGATTCTCACTCCAG
$p21^{Waf1/Cip}$	For: CAGCAGAGGAAGACCATGTG
	Rev: CGGCGTTTGGAGTGGTAG
Cdx-2	For: GCCAAGTGAAAACCAGGAC
	Rev: GGTGATGTAGCGACTGTAGTGA
Hes-1	For: CAACACGACACCGGATAAAC
	Rev: CGCGAGCTATCTTTCTTCAG
Msi-1	For: GGCAGACTACGCAGGAAGG
	Rev: TTCACGTCCTCCACCGTG

Table 9.2: Primers for PCR amplification of specific genes

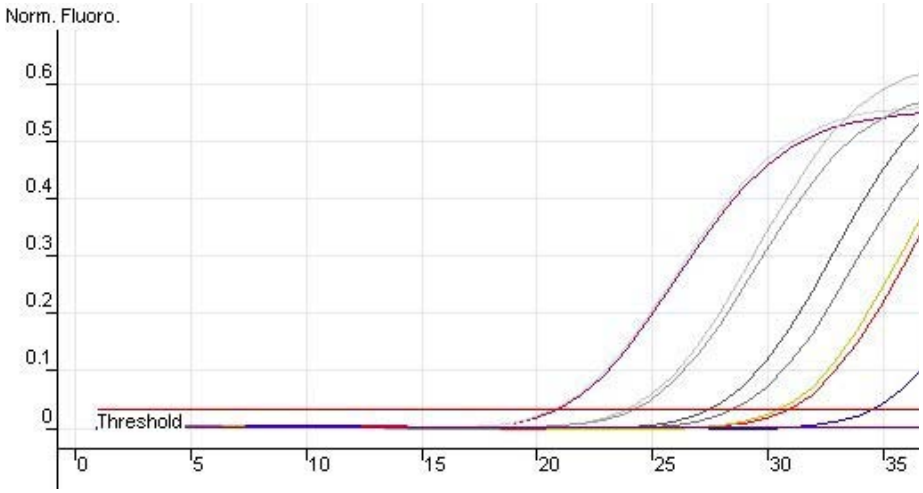

Figure 9.1: Example of Real-Time PCR results illustrating placement of the threshold value above any baseline activity and within the exponential increase phase. This represented the point at which gene expression levels were thus determined.

9.3: Results

9.3.1: Butyrate treatment reduced viability of both AGS and Caco-2 cells

The viability of AGS cells was dramatically decreased after both 24hr and 48hr incubation with all concentrations of butyrate compared to vehicle control values (Fig 9.2). Butyrate treatment was also observed to decrease Caco-2 cell viability after 24hr and 48hr incubation compared to vehicle control (Fig 9.3).

9.3.2: Gene expression studies

9.3.2.1: Bcl-2 (anti-apoptotic) expression was up-regulated by butyrate in AGS cells but down-regulated in Caco-2 cells

Butyrate treatment (1mM, 5mM and 10mM) led to an increase in expression of Bcl-2 after 24hr incubation in AGS gastric cancer cells compared to control values (Fig 9.4A). Bcl-2 expression was noted to further rise after 48hr incubation with butyrate in AGS cells. In contrast, Bcl-2 expression decreased in Caco-2 cells after 24hr and 48hr incubation with all concentrations of butyrate, compared to vehicle control (Fig 9.4B). No result is given for 48hr treatment with 5mM butyrate in Caco-2 cells due to insufficient sample volume.

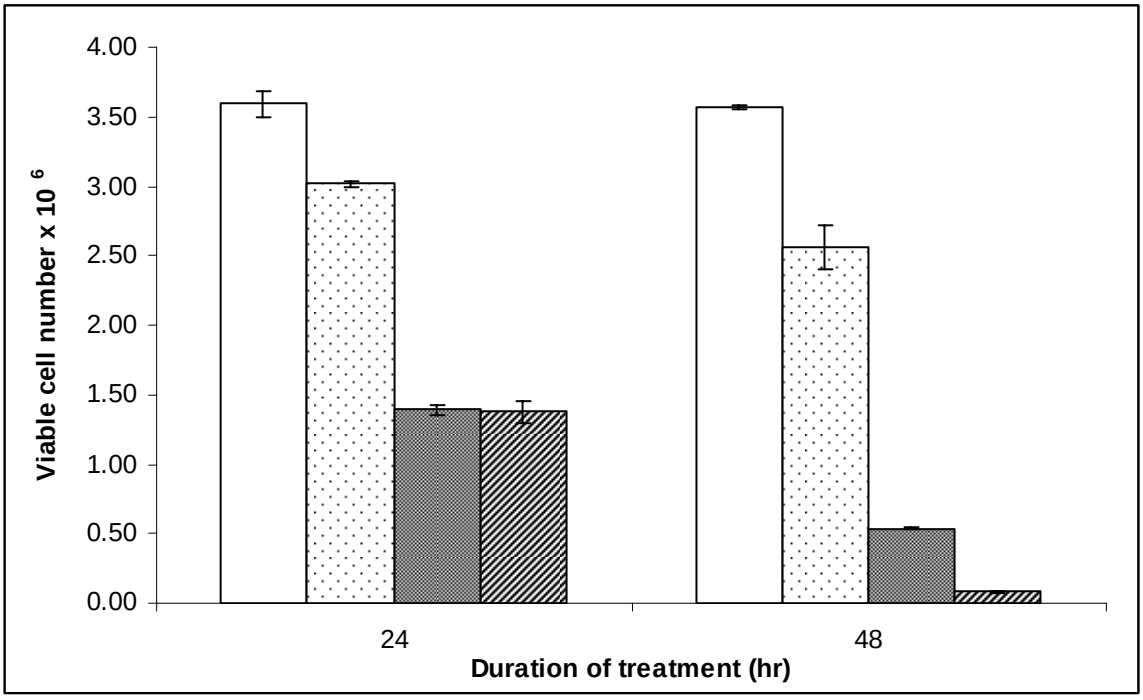

Figure 9.2: AGS viable cell number following 24hr and 48hr incubation with 1mM, 5mM and 10mM butyrate compared to vehicle control (n = 2). Data are presented as mean ± SEM (n = 2).

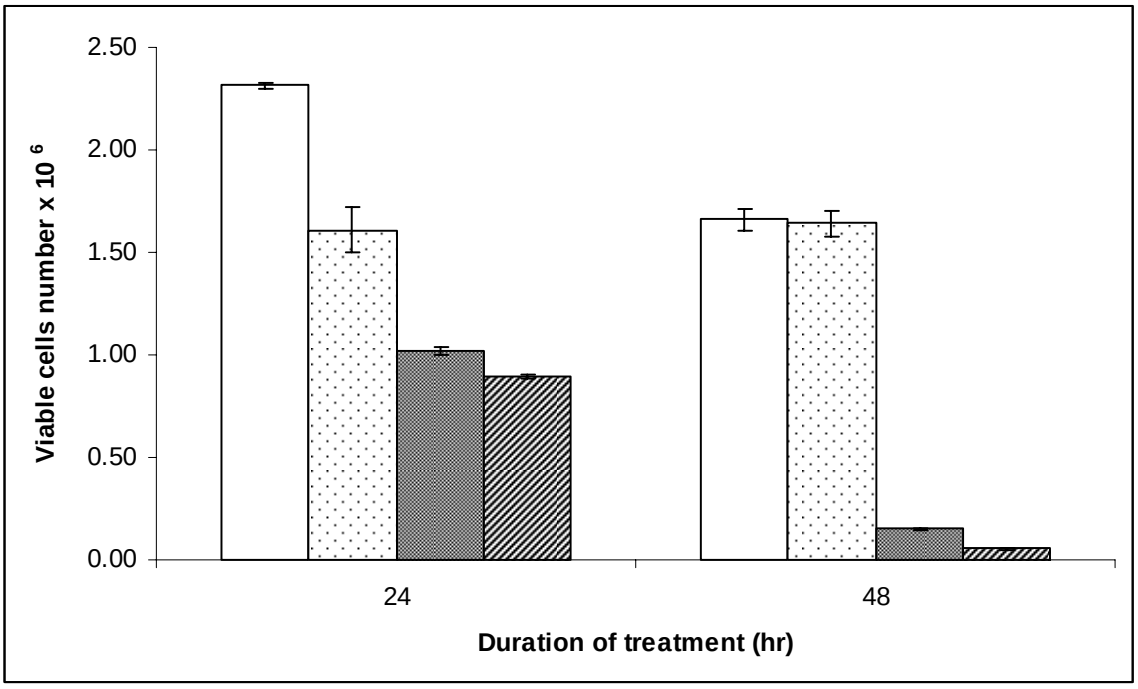

Figure 9.3: Caco-2 viable cell number following 24hr and 48hr incubation with 1mM 5mM and 10mM butyrate compared to vehicle control (n = 2). Data are presented as mean ± SEM (n = 2).

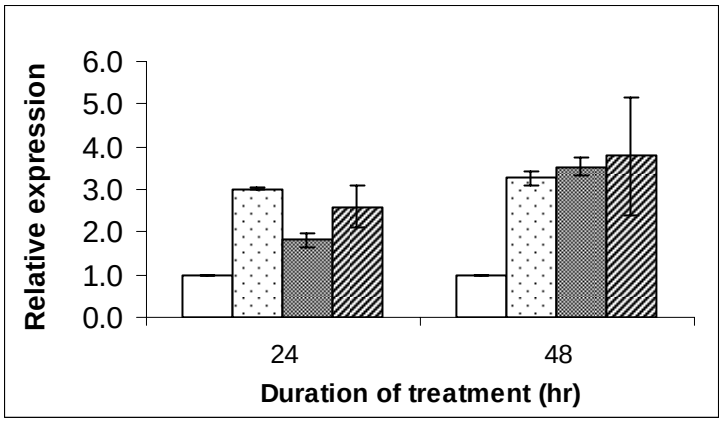

Fig 9.4A: Bcl-2 gene expression levels in AGS cells following 24hr and 48hr incubation with 1mM, 5mM, and 10mM butyrate compared to vehicle control. Data are presented as mean ± SEM (n = 2).

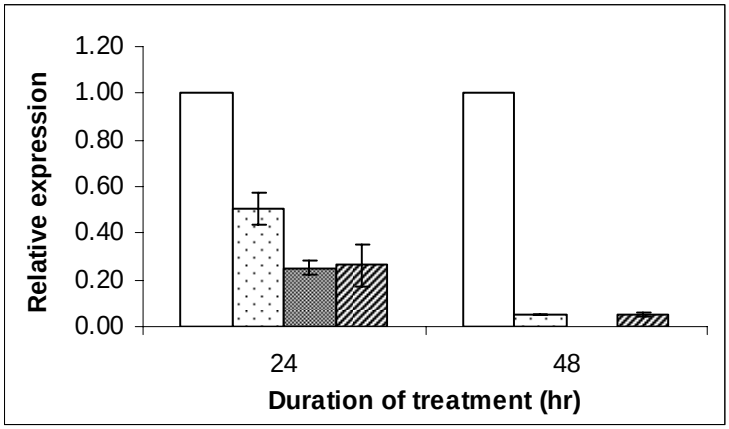

Fig 9.4B: Bcl-2 gene expression levels in Caco-2 cells following 24hr and 48hr incubation with 1mM, 5mM, and 10mM butyrate compared to vehicle control. Data are presented as mean ± SEM (n = 2). Note that no result is given for 48hr, 5mM butyrate due to lack of sufficient sample.

9.3.2.2: Butyrate treatment induced differential expression of Bcl-X_L (anti-apoptotic) in AGS and Caco-2 cells

Bcl-X_L expression was up-regulated in AGS cells after 24hr incubation with all concentrations of butyrate compared to control (Fig 9.5A). After 48hr butyrate treatment, no real differences in Bcl-X_L expression were observed, although overall Bcl-X_L expression levels were much less than at 24hr. However, a trend towards increased levels of Bcl-X_L expression can be noted with 1mM butyrate and decreased expression levels with 10mM butyrate compared to vehicle control. Figure 9.5B documents large decreases in Bcl-X_L x expression in Caco-2 cells after 24hr and 48hr treatment at all concentrations of butyrate compared to control.

9.3.2.3: Differential expression of Bax (pro-apoptotic) in AGS and Caco-2 cells following butyrate treatment

Bax was differentially expressed in AGS cells following treatment with butyrate (Fig 9.6A). Figure 9.6A shows an increase in Bax expression at 24hr incubation with 1mM butyrate and a decrease in its expression with 5mM and 10mM butyrate compared to control. After 48hr incubation, trends towards dose-dependent decreases in levels of Bax expression were observed in AGS cells. In Caco-2 cells, 24hr butyrate treatment (1mM, 5mM and 10mM) reduced the expression of Bax (Fig 9.6B). Incubation with 10mM butyrate appeared to reduce Bax expression after 48hr but not at 1mM or 5mM compared to control values.

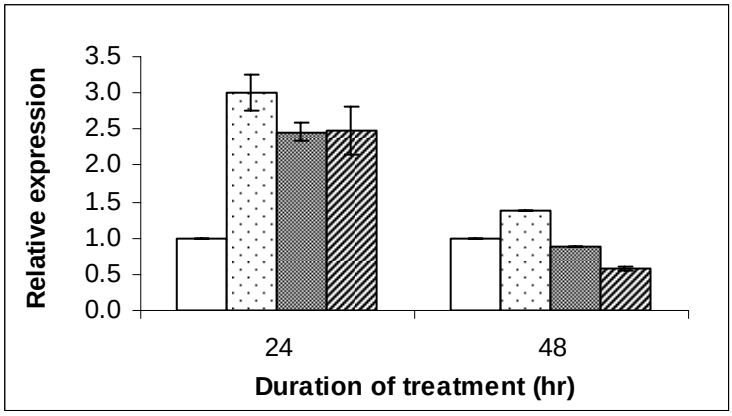

Fig 9.5A: Bcl-X_L gene expression levels in AGS cells following 24hr and 48hr incubation with 1mM, 5mM, and 10mM butyrate compared to vehicle control. Data are presented as mean ± SEM (n = 2).

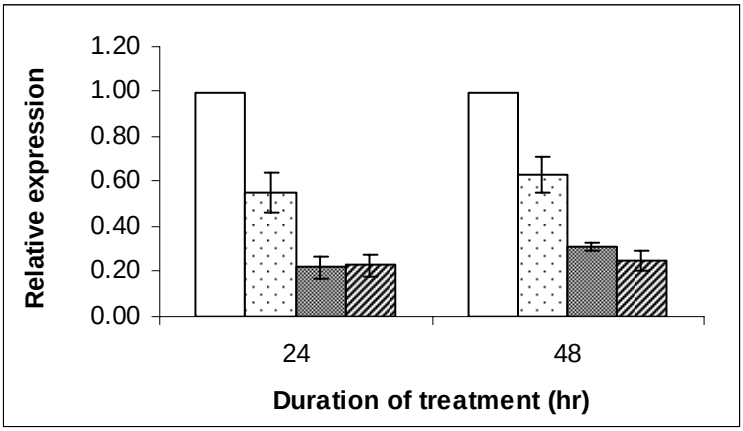

Fig 9.5B: Bcl-X_L gene expression levels in Caco-2 cells following 24hr and 48hr incubation with 1mM, 5mM, and 10mM butyrate compared to vehicle control. Data are presented as mean ± SEM (n = 2).

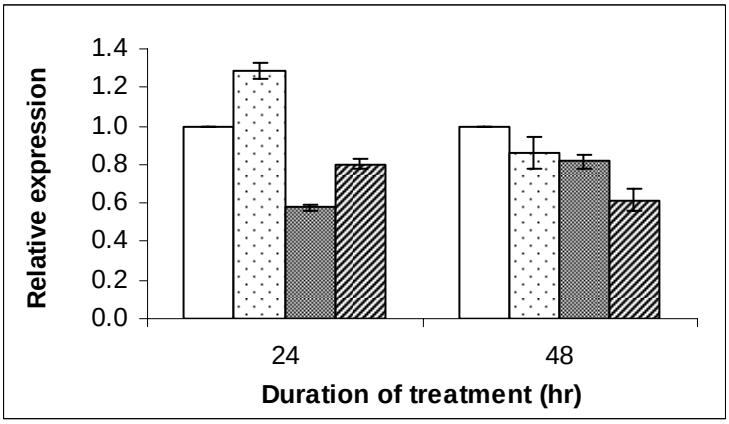

Fig 9.6A: Bax expression levels in AGS cells following 24hr and 48hr incubation with 1mM ⬚, 5mM ▩, and 10mM ▨ butyrate compared to vehicle control □. Data are presented as mean ± SEM (n = 2).

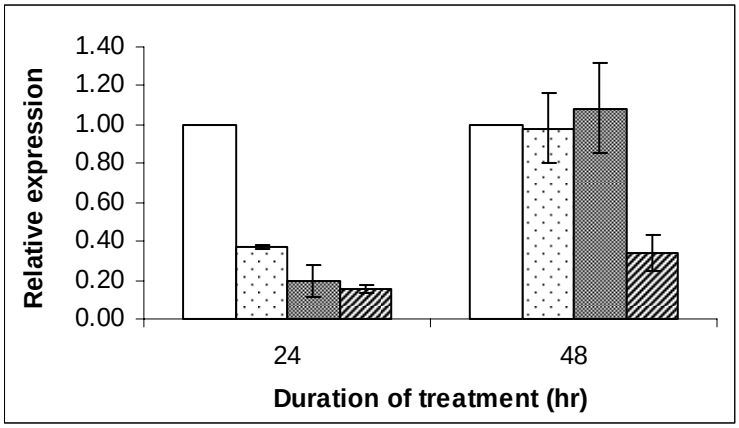

Fig 9.6B: Bax expression levels in Caco-2 cells following 24hr and 48hr incubation with 1mM ⬚ , 5mM ▩, and 10mM ▨ butyrate compared to vehicle control □. Data are presented as mean ± SEM (n = 2).

9.3.2.4: ODC expression was increased by butyrate in both AGS and Caco-2 cells

ODC expression was increased after 24hr and 48hr treatment with 5mM and 10mM butyrate compared to controls in AGS cells although 1mM butyrate appeared to reduce ODC expression at 48hr incubation (Fig 9.7A). All concentrations of butyrate (1mM, 5mM and 10mM) induced increases in the expression of ODC in Caco-2 cells after 24hr and 48hr compared to vehicle control values (Fig 9.7B).

9.3.2.5: Butyrate treatment induced $p21^{Waf1/Cip}$ expression in AGS and Caco-2 cells

Incubation with 5mM and 10mM butyrate induced increases in $p21^{Waf1/Cip}$ expression in AGS cells after 24hr and 48hr but not with 1mM butyrate compared to vehicle control values (Fig 9.8A). However, the increase in $p21^{Waf1/Cip}$ expression by butyrate treatment was noted to be much less after 48hr than 24hr incubation. The expression of $p21^{Waf1/Cip}$ increased in Caco-2 cells after 24hr incubation with all concentrations of butyrate (Fig 9.8B). Incubation with 5mM and 10mM butyrate for 48hr led to increases in $p21^{Waf1/Cip}$ expression in Caco-2 cells but not with 1mM butyrate compared to controls. In contrast to $p21^{Waf1/Cip}$ expression in AGS cells, the expression of $p21^{Waf1/Cip}$ was greater after 48hr than 24hr.

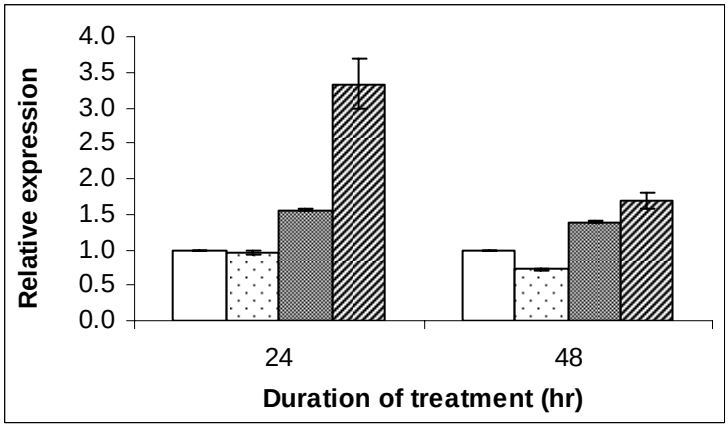

Fig 9.7A: ODC gene expression levels in AGS cells following 24hr and 48hr incubation with 1mM, 5mM, and 10mM butyrate compared to vehicle control. Data are presented as mean ± SEM (n = 2).

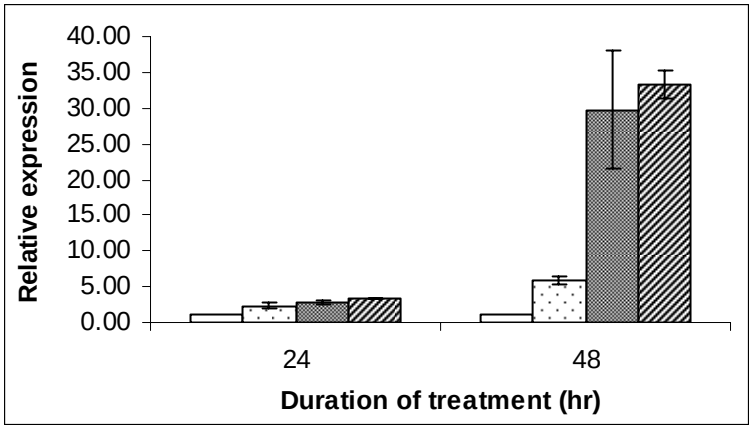

Fig 9.7B: ODC gene expression levels in Caco-2 cells following 24hr and 48hr incubation with 1mM, 5mM, and 10mM butyrate compared to vehicle control. Data are presented as mean ± SEM (n = 2).

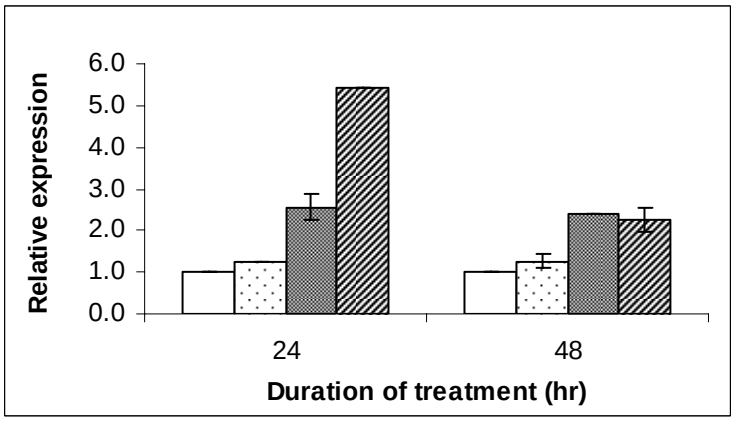

Fig 9.8A: p21$^{Waf1/Cip}$ gene expression levels in AGS cells following 24hr and 48hr incubation with 1mM ⬚, 5mM ▩, and 10mM ▨ butyrate compared to vehicle control □. Data are presented as mean ± SEM (n = 2).

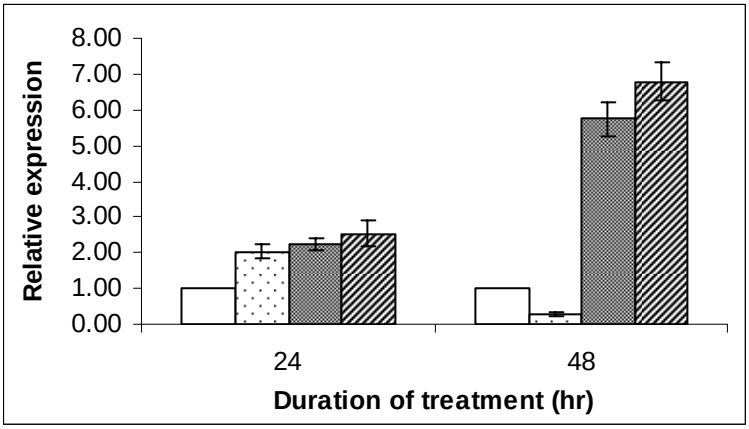

Fig 9.8B: p21$^{Waf1/Cip}$ gene expression levels in Caco-2 cells following 24hr and 48hr incubation with 1mM ⬚, 5mM ▩, and 10mM ▨ butyrate compared to vehicle control □. Data are presented as mean ± SEM (n = 2).

9.3.2.6: Butyrate treatment induced differential expression of Cdx-2 in AGS and Caco-2 cells

A minor increase in Cdx-2 expression in AGS cells was observed after 24hr incubation with 10mM butyrate but not with 1mM or 5mM compared to control (Fig 9.9A). After 48hr incubation with all concentrations of butyrate, Cdx-2 expression appeared to be increased to a greater extent than after 24hr compared to control values. Cdx-2 expression in Caco-2 cells was greatly decreased after 24hr and 48hr incubation with all concentrations of butyrate compared to vehicle control (Fig 9.9B).

9.3.2.7: Butyrate treatment differentially induced Hes-1 expression in AGS and Caco-2 cells

Incubation of AGS cells with 1mM butyrate led to an increase in Hes-1 expression compared to control values although no differences were observed with 5mM and 10mM butyrate (Fig 9.10A). Interestingly, 48hr incubation of AGS cells with 5mM and 10mM butyrate, but not 1mM butyrate, induced decreases in Hes-1 expression compared to control. In Caco-2 cells, incubation with all concentrations of butyrate induced increases in Hes-1 expression after 24hr compared to control (Fig 9.10B). After 48hr incubation, Hes-1 expression in Caco-2 cells was increased with 5mM and 10mM butyrate but not with 1mM butyrate compared to control values.

9.3.2.8: Msi-1 expression was down-regulated by butyrate in Caco-2 cells

A decrease in msi-1 expression in Caco-2 cells was observed after 24hr and 48hr incubation with 1mM, 5mM and 10mM butyrate compared to vehicle control values (Fig 9.11).

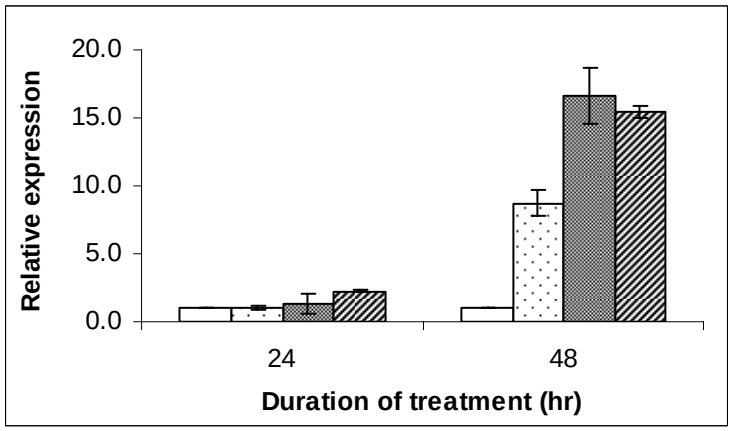

Fig 9.9A: Cdx-2 gene expression levels in AGS cells following 24hr and 48hr incubation with 1mM, 5mM, and 10mM butyrate compared to vehicle control. Data are presented as mean ± SEM (n = 2).

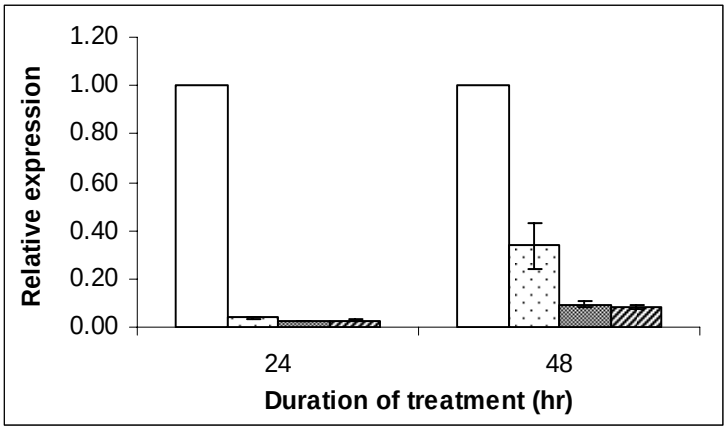

Fig 9.9B: Cdx-2 gene expression levels in Caco-2 cells following 24hr and 48hr incubation with 1mM, 5mM, and 10mM butyrate compared to vehicle control. Data are presented as mean ± SEM (n = 2).

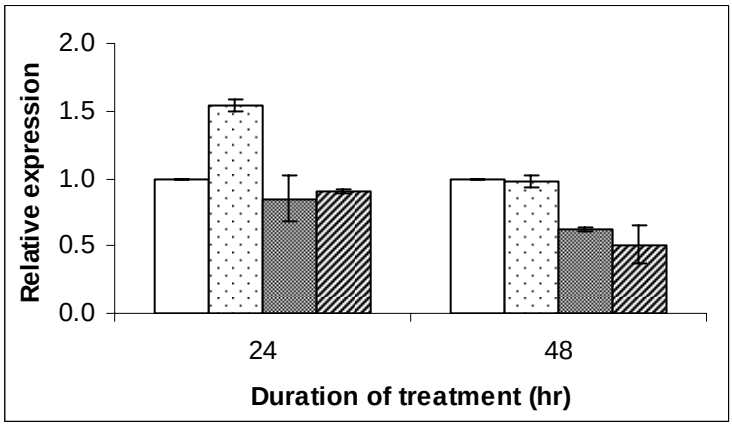

Fig 9.10A: Hes-1 gene expression levels in AGS cells following 24hr and 48hr incubation with 1mM, 5mM, and 10mM butyrate compared to vehicle control. Data are presented as mean ± SEM (n = 2).

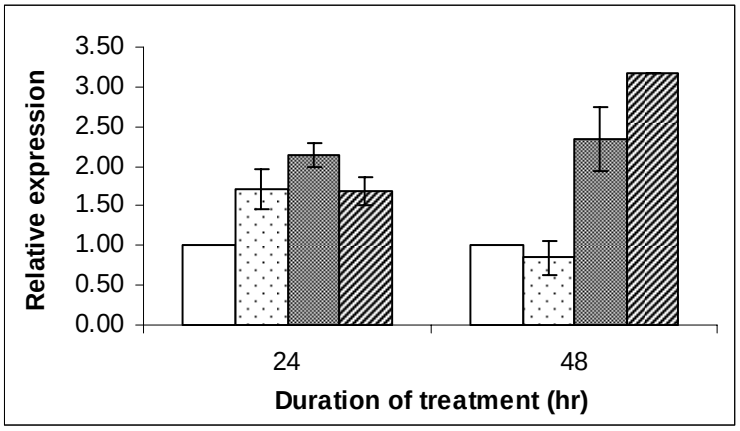

Fig 9.10B: Hes-1 gene expression levels in Caco-2 cells following 24hr and 48hr incubation with 1mM , 5mM , and 10mM butyrate compared to vehicle control . Data are presented as mean ± SEM (n = 2).

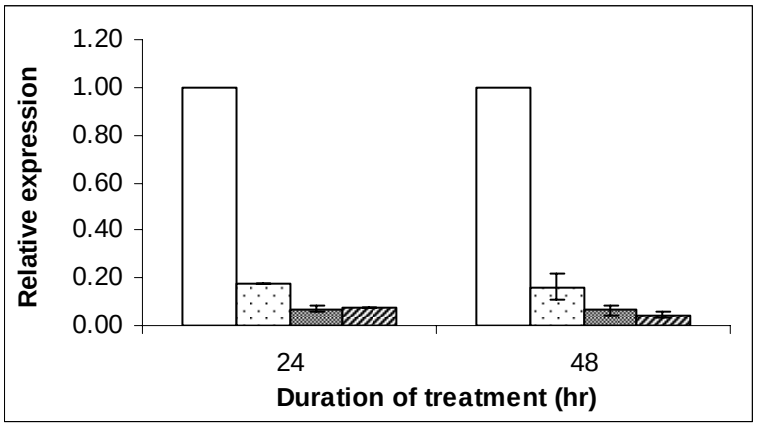

Fig 9.11: Msi-1 gene expression levels in Caco-2 cells following 24hr and 48hr incubation with 1mM, 5mM, and 10mM butyrate compared to vehicle control. Data are presented as mean ± SEM (n = 2).

9.4: Discussion

The current pilot study was a preliminary investigation into the effects of butyrate treatment on the expression of a number of specific candidate genes in a gastric adenocarcinoma cell line, AGS, and a colon carcinoma cell line, Caco-2, using Real-Time PCR. The genes were selected due to their likely associations with the effects of butyrate, including its ability to induce apoptosis and cellular differentiation [11, 82, 117]. These genes included: Bcl-2 (anti-apoptotic), Bcl-X_L (antiapoptotic), Bax (proapoptotic), ODC (cell proliferation marker), $p21^{Waf1/Cip}$ (cell cycle regulator), Cdx-2 (developmental regulator), Hes-1 (differentiation) and Msi-1 (proposed stem cell marker). It was observed that butyrate induced differential changes to the expression of many of the genes assessed in AGS and Caco-2 cells that were associated with decreases in viability of both cell lines.

9.4.1: Apoptosis-associated gene expression

The present study observed a number of differences in butyrate-modulated expression of the apoptosis-associated genes, Bcl-2, Bcl-X_L and Bax, between AGS cells and Caco-2 cells. This was an interesting finding as butyrate appeared to induce very similar decreases in cell viability in both cell lines within this pilot study.

The upregulation of Bcl-2 and Bcl-X_L is reported to inhibit the induction of apoptosis though the prevention of cytochrome C release from mitochondria [14, 119]. In contrast, overexpression of Bax has been demonstrated to promote the onset of apoptosis by promoting mitochondrial damage and cytochrome C release [120-122]. Therefore, the decreases in Bcl-2 and Bcl-X_L expression, but not Bax, in Caco-2 cells inferred that butyrate-mediated decreases in cell viability may have been due to the induction of cell death, particularly by apoptosis, through a

mitochondrial damage pathway. It was also proposed that the concomitant reduction in Bax expression in Caco-2 cells may have signified an opposing cellular response in order to prevent cell death by butyrate. In contrast to the expression levels evident in Caco-2 cells, Bcl-2 and Bcl-X_L were up-regulated in AGS cells, at least initially, by butyrate treatment while Bax expression was partly down-regulated. Indeed, this gene expression profile would normally be associated with a non-apoptotic phenotype. Therefore, these differences in expression levels suggested that the pathway leading to the induction of cell death by butyrate was different between the two cell lines. Moreover, it appeared that butyrate-induced cell death in Caco-2 cells was mediated via a Bcl-2-dependent pathway while cell death in AGS cells was mediated via a Bcl-2-independent pathway.

9.4.2: ODC and $p21^{Waf1/Cip}$ expression

The current study demonstrated that the levels of ODC expression in AGS and Caco-2 cells reflected that of $p21^{Waf1/Cip}$ expression, although not between the cell lines. Furthermore, an increase in expression of both ODC and $p21^{Waf1/Cip}$ occurred earlier (24hr) in AGS cells than Caco-2 cells (48hr). This is interesting as ODC is known to be a positive marker of cellular proliferation (Seiler and Raul, 2005), whereas $p21^{Waf1/Cip}$ is known to be a cell cycle regulator, whereby increases in its levels are reflected by arrest of the cell cycle [86, 129]. We have previously shown that Caco-2 cells undergo cell cycle arrest in G2-M phase following butyrate treatment (Chapter 6) potentially due to the up-regulation of $p21^{Waf1/Cip}$ expression, as suggested by this pilot study. Butyrate is also known to induce differentiation in transformed cells (and thus the observed induction of ODC expression suggested that this may have also occurred within the cells studied. The assessment of gene expression within this study was undertaken in a mix of both viable and non-viable cells due to the sampling method utilised. Thus, the parallel expression levels of ODC and $p21^{Waf1/Cip}$ may have represented the levels within viable and non-

viable cells, respectively. It is suggested that future studies should separate viable from non-viable cells prior to assessment of expression levels of specific genes following butyrate treatment.

9.4.3: Cdx-2 expression

The present study assessed the expression levels of an important gene thought to regulate the development and homeostasis of the gut, Cdx-2 [131]. We observed that butyrate treatment up-regulated expression of Cdx-2 in AGS gastric adenocarcinoma cells. This was an interesting finding as adenocarcinoma cells were thought to represent a more normal cell type than carcinoma cells [133]. Indeed, Qualtrough *et al*. [133] reported the lack of any regulation of Cdx-2 by butyrate in six colorectal adenoma cell lines. Potentially, this suggested that butyrate may have induced differentiation, acting as a tumour suppressor in AGS gastric adenocarcinoma cells by up-regulating Cdx-2 expression and inducing cell death mechanisms. This was highlighted by the concomitant reduction in cell viability in this cell type. Potentially, this suggested that Cdx-2 expression was regulated differently in gastric adenoma cells than in gastric adenocarcinoma cells, which may be related to their differentiation status.

In contrast, Cdx-2 expression by butyrate was down-regulated in Caco-2 colon carcinoma cells. This result opposes the up-regulated expression of Cdx-2 by butyrate previously demonstrated in Caco-2 colon carcinoma cells [131]. However, we are unaware of any other studies that have shown up-regulated Cdx-2 expression by butyrate. Indeed, the laboratory in which these studies were undertaken had previously shown down-regulation of Cdx-2 by butyrate in Caco-2 cells (personal communication: Dr Jim Wilson). It is suggested, therefore, that changes to Cdx-2 expression may be cell type specific. Furthermore, clonal differences within a cell line, such as Caco-2, and differentiation status may also induce differences in Cdx-2 expression, and its response to butyrate treatment.

9.4.4: Hes-1 expression

The current pilot study showed an induction of Hes-1 expression by butyrate in Caco-2 cells in parallel with the report by Stockhausen *et al*. [138] who assessed the effects of valproic acid. This inferred that butyrate acted, at least in part, through the Notch signalling pathway to induce cellular differentiation and cell death in Caco-2 cells. Moreover, Hes-1 activation of the Notch pathway may act in a tumour suppressive fashion in this cell line.

In contrast, butyrate was demonstrated to differentially effect Hes-1 expression in AGS cells. Specifically, after short-term incubation (24hr) the lowest butyrate concentration (1mM) induced an up-regulation of Hes-1 expression, whereas longer-term incubation (48hr) with higher concentrations of butyrate (5mM and 10mM) reduced its expression. This suggested that in the short-term, butyrate may have acted through a Hes-1/Notch signalling pathway, but only at low concentrations in AGS cells. However, as treatment duration continued, the higher concentrations of butyrate induced a down-regulation of Hes-1/Notch signalling suggesting that the apoptotic actions of butyrate were potentially mediated through a different pathway under these conditions. Hence, Hes-1/Notch signalling may act as an oncogene in this gastric cancer cell line. Indeed, Notch signalling is reported to have opposing effects in different cancer cell types.

9.4.5: Msi-1 expression

Msi-1 is documented as a stem cell marker within the gastrointestinal tract [143, 147]. Consistent with our hypothesis, butyrate was observed to reduce Msi-1 expression in Caco-2 cells. We are unaware of any studies that have reported this modulation of Msi-1 expression by butyrate, particularly in this cell type, except from within the laboratory in which this study was undertaken (unpublished observations). Therefore, the results of this pilot study suggested that

differentiation-associated effects of butyrate may have been mediated through the down-regulation of Msi-1. Interestingly, however, Msi-1 did not appear to be a positive regulator of Hes-1 expression, as suggested by one report [148], due to the opposing up-regulation of Hes-1 observed within this study.

9.4.6: Conclusions

The current study has provided preliminary evidence for the differential expression of a number of genes in AGS and Caco-2 cells by butyrate. It must be noted, however, that the changes to gene expression within this pilot study were determined from only two samples and hence, should only be regarded as an estimate of the effects of butyrate. However, it appears likely that distinct, cell-line-specific differences exist in the effects of butyrate on gene expression that ultimately lead to cell death in both cancer cell lines. It is proposed that this may be due to differences in the handling of butyrate by both cancer cell types, particularly in its metabolism. Therefore, further investigations are required in order to effectively determine the mode of action of butyrate between different cell lines.

CHAPTER 10: DISCUSSION/ CONCLUSIONS

10.1: Introduction

It is currently accepted that butyrate plays a major role in the prevention of colonic neoplasms [79, 103, 105, 110] and the maintenance of colonic homeostasis [33, 70, 103, 153]. However, its mode of action is not completely understood, and even less is known about the effects of the SCFA, propionate. Recently, the potential for butyrate to treat other human neoplasms, such as lymphoma [41, 115] and leukaemia, [154] has been reported. This led our laboratory to investigate the potential for butyrate and propionate to serve as a treatment for gastric cancer.

The current thesis investigated the *in vitro* modulation of apoptosis by the SCFAs, butyrate and propionate, in gastric cancer and colon cancer cell lines. Specifically, this thesis assessed the induction of apoptosis and cell cycle alterations by butyrate and propionate combined with measurements of OPP activity, GSH availability and glucose oxidation. It was determined that both butyrate and propionate led to the induction of apoptosis in both cell lines, however colon cancer cells appeared to have a significantly greater sensitivity to both SCFAs than the gastric cancer cell line. Moreover, the level of butyrate-induced apoptosis was demonstrated to be strongly associated with differential rates of butyrate metabolism between the two cell lines. Additionally, it was demonstrated that butyrate and propionate induced apoptosis, at least in part, by the modulation of intracellular redox status and cellular protection mechanisms.

10.2: Development of a novel measure of glucose oxidation

This thesis describes a new technique for the measurement of G6PDH activity and ^{13}C-glucose oxidation in Kato III gastric cancer cells. This technique allows the concomitant assessment of

OPP activity and D-glucose metabolism in cell culture enabling the estimation of glucose flux profiles to be made following changes to the cellular environment, drug administration or gene expression. A novel CO_2 collection chamber was also developed that could be easily utilised within any cell culture laboratory for the assessment of cellular metabolism, including D-glucose oxidation and butyrate metabolism.

Chapter 2 describes the use of this new technique for the assessment of D-glucose metabolism in Kato III cells following a) serum-deprivation, b) incubation with the OPP inhibitor, DHEA, or c) incubation with IGF-I. It was demonstrated that serum-deprivation initially led to parallel decreases in glucose oxidation and OPP activity. However, after 24hr serum-starvation, a divergence in OPP activity and D-glucose metabolism was observed. OPP activity was noted to return to non-starved levels whereas D-glucose oxidation had become greatly up-regulated compared to baseline. This may have indicated an initial period of low metabolic activity due to serum withdrawal followed by a period of increased energy production by the TCA cycle within this cell line.

The incubation of Kato III cells with DHEA following serum deprivation was demonstrated to significantly reduce G6PDH activity and D-glucose oxidation after short-term (2hr) incubation. However, after longer-term incubation (24hr), both parameters returned to non-treated levels, most likely due to the complete utilisation of DHEA by this time point. Under normal conditions, D-glucose is reported to be preferentially metabolised through the OPP [71]. Therefore, in agreement with other studies [21, 22], it was demonstrated that the OPP was rapidly inhibited by DHEA and that this led to a reduction in D-glucose flux, potentially through this pathway.

IGF-I has been shown to induce increases in the flux of glucose through glycolysis and the TCA cycle in a vascular smooth muscle cell line [24]. It has also been reported to stimulate the proliferation of AGS gastric cancer cells [75]. The current study, however, described a reduction in glucose metabolism by IGF-I away from both the OPP and the TCA cycle in serum-deprived Kato III cells. This suggested that treatment of the Kato III gastric cancer cell line with IGF-I led to a reduction in the requirement of this cell type for glucose oxidation. This infers an IGF-I-induced reduction in the fuel requirements of these serum-starved cells.

As a result of these findings, a new technique has been described and validated that allows the rapid assessment of D-glucose metabolism in Kato III cells that could be easily utilised with many other cell lines. It is proposed that this method could provide a tool for profiling shifts in glucose metabolism in target cells during drug development, and could aid in the future identification of novel anti-cancer agents. This technique was utilised throughout the remainder of this thesis to profile glucose and butyrate metabolism in the Kato III and Caco-2 cell lines following treatment with various agents, including SCFAs.

10.3: Modulation of apoptosis by SCFAs in Kato III and Caco-2 cells

In Chapter 3 it was demonstrated that butyrate and propionate induced significant increases in the total level of apoptosis in Kato III cells. Moreover, butyrate treatment led to greater reductions in cell viability than propionate. Indeed, a maximal reduction in cell viability of 14% by 5mM butyrate was observed compared to an 8% reduction by 10mM propionate, after 72hr incubation, in this cell line. Interestingly, the current study reported that the 5mM combination SCFA treatment caused significantly greater apoptosis-induction than an identical concentration of either SCFA alone, but only after long-term incubation (72hr). This implies that a butyrate-

propionate combination treatment has the potential to be utilised in the treatment of gastric cancer with greater efficacy than either SCFA alone.

The current study also demonstrated that the type of cell death (apoptosis or necrosis) induced within Kato III cells was dependent on the SCFA utilised. Jeng *et al*. [83] suggested that butyrate induced cell death by mechanisms other than apoptosis alone. Indeed, in Kato III cells it was observed that butyrate treatment induced both apoptosis and necrosis at all time points. In contrast, propionate induced cell death mainly through the induction of apoptosis, except within the first 24hr of treatment. Thus, it was concluded that the greater sensitivity of Kato III cells to butyrate was, in part, due to its ability to induce both apoptosis and necrosis.

In Chapter 6, the effects of SCFAs on the viability of Caco-2 cells were investigated. In parallel with Kato III cells, it was demonstrated that butyrate induced significantly greater reductions in cell viability (decreased by 49%) than propionate (decreased by 26%) in the Caco-2 cell line. However, in contrast to Kato III cells, the Caco-2 cells within this study exhibited a dose-dependent response to butyrate, whereby cell viability was reduced to the greatest extent by the highest concentration of butyrate (10mM). Additionally, the ability of butyrate to induce apoptosis in Caco-2 cells was significantly reduced when combined with propionate (i.e. 5mM combination SCFA treatment, 31% increase vs. 36% butyrate alone), in contrast to that observed with Kato III cells.

When the mode of cell death by each SCFA was assessed in Caco-2 cells, it was demonstrated that butyrate alone, and the SCFA combination treatment induced necrosis to a greater extent than propionate alone, in agreement with the findings in Kato III cells. This is an interesting finding as previous studies have only reported the ability of butyrate to induce apoptosis, and not

necrosis, in various cell types [11, 114]. Therefore, the enhanced sensitivity of the Caco-2 and Kato III cell lines to butyrate compared to propionate appears due, at least in part, to its necrosis-inducing properties. However, these properties of butyrate do not completely explain its enhanced ability to induce cell death as it also induced significantly greater increases in the proportion of overall apoptosis compared with propionate in Caco-2 cells. Interestingly, the combination SCFA treatment induced a significantly greater percentage of necrosis than butyrate alone. This may have been due to the combination treatment being physiologically similar to the SCFA composition of the normal colon, where both SCFAs are produced. Thus, a combination treatment may produce similar degrees of apoptosis and necrosis *in vitro* with that seen *in vivo*. Therefore, future studies should utilise combined SCFA treatments, particularly when investigating their effects on colon-derived cell lines.

One of the main hypotheses of this thesis was that a gastric cancer cell line would be more sensitive than a colon cancer cell line to the apoptosis-inducing effects of SCFAs. Prior to discussion of this it must be noted that although Kato III cells were treated with SCFAs for up to 72hr, Caco-2 cells were only treated up to 48hr. This was due to the significantly greater incubation times required for Caco-2 cells compared to Kato III cells prior to treatment. Therefore, all comparisons between cell lines were conducted at the 48hr time point.

In chapter 3, butyrate and propionate increased the induction of apoptosis to a maximum level of only 6.0% and 5.5% in Kato III cells, respectively, after 48hr incubation. This was in comparison to a maximum induction of TA in Caco-2 cells of 35.6% and 15.8% by butyrate and propionate at the same time point, respectively, (Chapter 6). Moreover, in terms of overall cell death induced by butyrate (including necrosis), the maximal reduction in viability of Kato III cells was 14.4% (5mM butyrate), whereas in Caco-2 cells it was reduced by 48.5% (10mM butyrate).

Therefore, Caco-2 cells exhibited a significantly greater sensitivity to SCFA treatment than Kato III cells, in opposition to the original hypothesis. Hence, it was demonstrated that cell-line specific differences exist that alter the effects of SCFA treatment. This may have been due to differences in the ability of each cell type to induce apoptosis signalling pathways, such as p53.

p53 is a tumour suppressor gene that responds to DNA damage by inducing cell cycle arrest and apoptosis [3, 155, 156] (Figure 10.1). However, the Kato III cell line does not contain a functional p53 gene, and hence the induction of apoptosis in this cell type would occur via a p53-indepenent pathway. Indeed, it has been previously described that approximately half of all human cancers contain p53-mutations [3], with reports of up to 87% in gastric cancers [157, 158]. In contrast, the Caco-2 cell line contains a functional p53 gene.

Recently, the induction of apoptosis by butyrate has been described mainly via a p53-dependent pathway [112, 116]. Takimoto *et al*. [112] observed that butyrate treatment exerted a small reduction in growth of wild-type (p53-null) Kato III cells similar to that described within the current thesis. However, when Kato III cells were transfected with wild-type p53, and subsequently treated with butyrate, a dramatic reduction in cell viability was observed. By 72hr incubation with butyrate, no viable Kato III cells containing the functional p53 gene were detected. Moreover, the induction of apoptosis by butyrate in Kato III cells transfected with the p53-wild type gene was increased almost fifty fold from the levels apparent in non-transfected, butyrate treated Kato III cells. Therefore, it appears that p53 plays a major role in butyrate-induced cell death and this could most likely explain the demonstrated differences in sensitivity of Kato III and Caco-2 cells to butyrate within the current thesis. However, this thesis has described further differences in the effects of butyrate and/or propionate that may also partly explain the differences in sensitivities between each cell line. These will be discussed herein.

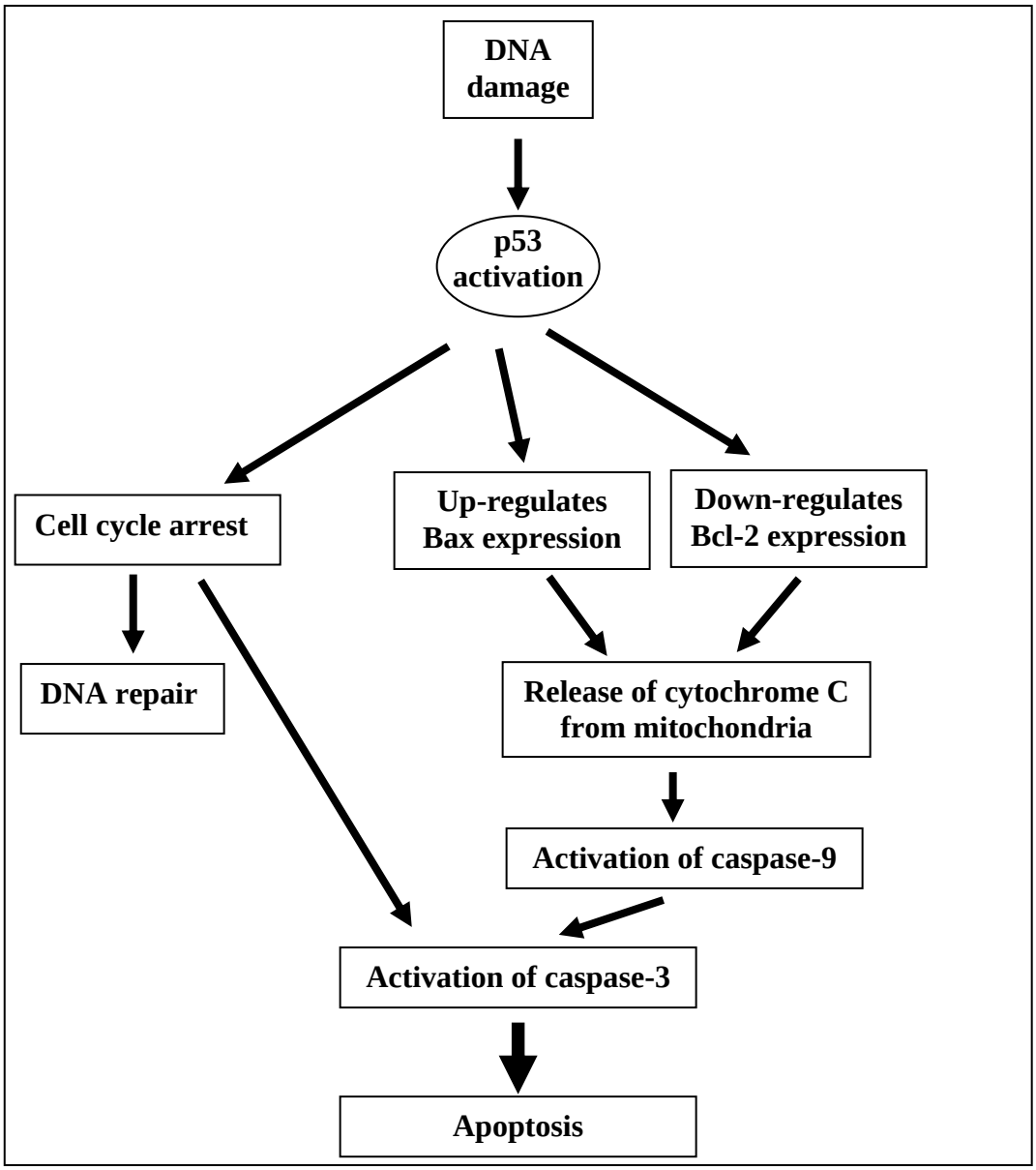

Figure 10.1: Intrinsic p53 apoptosis pathway: p53 is activated by DNA damage leading to arrest of the cell cycle followed by DNA repair or apoptosis. p53 also up-regulates the expression of pro-apoptotic, Bax, and the down-regulation of anti-apoptotic, Bcl-2. This allows the release of cytochrome C from mitochondria, the activation of caspase-9, and the eventual activation of down-stream caspase-3, leading to the induction of apoptosis.

10.4: Regulation of cell cycle by SCFA in Kato III and Caco-2 cells

In Chapter 3 and Chapter 6, dose-dependent decreases in the proportion of G0-G1 phase cells (Kato III vs. Caco-2; 11.5% vs. 22.1%) and increases in S (4.4% vs. 1.0%) and G2-M (7.2% vs. 19.6%) phase cells by 48hr butyrate treatment in Kato III and Caco-2 cells, were demonstrated. Interestingly, when the proportion of cells in each phase was compared at the earlier time point (24hr), it was observed that the progression through S phase and into G2-M phase occurred earlier in Caco-2 cells than in Kato III cells. Therefore, in contrast to many reports of G0-G1 phase arrests by butyrate, this thesis has described butyrate-induced alterations to the cell cycle leading to arrest in G2-M phase in both Kato III and Caco-2 cells. More importantly, the increased induction of G2-M arrest in Caco-2 cells than in Kato III cells suggests that it may be, in part, by this mechanism that a greater induction of cell death is achieved by butyrate.

Another interesting observation from the current study were the differential changes to the cell cycle in Caco-2 and Kato III cells by propionate treatment. In Caco-2 cells, propionate treatment led to significant dose-dependent reductions in G0-G1 cells (reduced by 16.9%) and increases in S phase (increased by 2.3%) and G2-M phase (increased by 13.9%) cells. At the same time point (48hr), propionate induced decreases in G0-G1 phase (reduced by 14.3%) and S phase (reduced by 4.2%) and increased G2-M phase (increased by 18.7%) in Kato III cells. Potentially, this indicated that propionate had a significantly greater ability to induce cell cycle changes and arrest in G2-M in Kato III cells than in Caco-2 cells. However, this was not translated into its ability to induce cell death in the Kato III cell line, as described previously. This suggested that arrest of the cell cycle in G2-M is not the primary cause of cell death by propionate in Kato III cells. In agreement, a recent study reported that arrest of the cell cycle in S phase or G2-M was not always associated with cell death [159].

The induction of cell cycle arrest has been demonstrated to involve several molecules, particularly p53, which is known to modulate apoptosis (described previously; Figure 10.1) and cell cycle arrest in response to DNA damage [160, 161]. Therefore, p53-null Kato III cells would be forced to regulate the cell cycle via a p53-independent pathway. In agreement, a recent study documented the potentiation of Fas-mediated apoptosis, an extrinsic cell death pathway, by butyrate in a transformed human keratinocyte cell line [162].

The membrane bound Fas (CD95/APO-1) protein belongs to the tumour necrosis factor (TNF) receptor family of membrane bound proteins [78, 163]. The activation of Fas is reported to lead to the recruitment of the adapter protein Mort1 to the Fas death domain (FADD). This ultimately leads to the activation of caspase-8 and the initiation of caspase-mediated apoptosis. Daehn *et al*. [162] demonstrated that butyrate-induced apoptosis in keratinocytes was mediated via an extrinsic pathway due the exclusive activation of caspase-3, but not caspase-9 (involved in intrinsic cell death pathways). Moreover, these investigators reported increases in surface expression of Fas by butyrate treatment, further supporting its role in butyrate-mediated cell death.

Fas has also been demonstrated to regulate non-apoptotic activities, including cell cycle progression [164-166]. Matsuyoshi *et al*. [165] reported that FADD plays an essential role in G2-M phase arrest in breast cancer cells. In addition, FADD-mediated cell cycle arrest was observed to occur in both a p53-dependent and a p53-independent manner. No assessment of Fas expression or FADD activation was undertaken within the current thesis. However, Atten *et al*. [78] recently reported the expression of Fas and Fas-Ligand (Fas-L) in Kato III cells. These investigators reported that resveratrol, a novel chemotherapeutic agent, induced cell death and cell cycle arrest through up-regulation of Fas-L alone in this cell line. In contrast, resveratrol up-

regulated both Fas and Fas-L expression in a colon cancer cell line containing the wild-type p53 gene. This suggested that in a cell line containing p53, such as Caco-2, cell death by butyrate may be activated via both a p53-dependent pathway and the Fas-mediated pathway (Figure 10.2). In contrast, butyrate-mediated cell death may only be activated via the Fas pathway in p53-deficient cells, such as Kato III (Figure 10.2). In turn, this could contribute to the overall level of apoptosis induction. Therefore, it is proposed that differences in the butyrate-induced activation of apoptotic pathways (Fas and/or p53) between Kato III and Caco-2 cell lines, may have explained the observed differences in the induction of cell death and cell cycle arrest, demonstrated within the current thesis. Further studies are required to effectively assess this hypothesis.

10.5: Modulation of OPP activity and GSH availability in Kato III and Caco-2 cells by butyrate and propionate

In Chapter 4, GSH availability was significantly reduced in Kato III cells following incubation with all concentrations of butyrate and propionate, in agreement with recent studies [49, 51]. It appeared that the reduction in GSH availability by both SCFAs in this cell line was time-dependent with the maximal change from control levels occurring after short-term (24hr) treatment. Interestingly, propionate reduced GSH availability to a greater extent than butyrate alone and the combination SCFA treatment in Kato III cells. This, however, is in opposition to the enhanced level of cell death induced by butyrate compared to propionate in Kato III cells.

Chapter 7 documented the modulation of GSH by SCFAs in Caco-2 cells. In parallel with the results in Kato III cells, it was observed that in Caco-2 cells, both butyrate and propionate treatment led to significant reductions in GSH availability. Specifically, it was demonstrated that the higher concentrations of butyrate (5mM and 10mM) and all concentrations of propionate

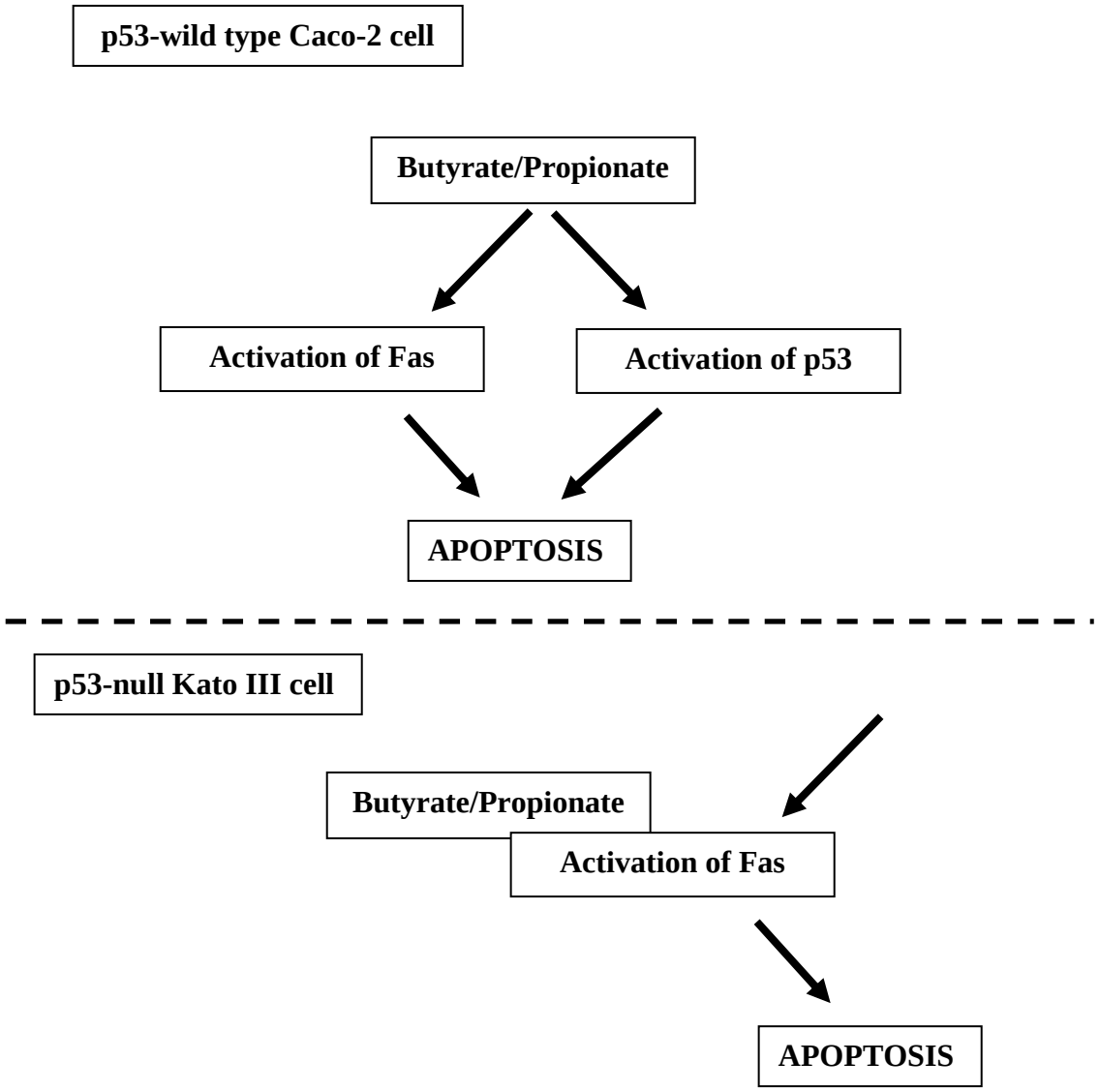

Figure 10.2: Schematic diagram depicting only the Fas and p53 pathways of apoptosis induction in the Caco-2 cell line, and the p53-null, Kato III cell line. It is proposed that at least two pathways (Fas and p53) will be forcing the induction of apoptosis by SCFAs in Caco-2 cells. In contrast, SCFA-induced apoptosis in Kato III cells is proposed to occur via the activation of Fas alone.

reduced GSH availability in Caco-2 cells at both time points assessed. Also, in agreement with the effects of SCFAs in Kato III cells, propionate reduced GSH availability in Caco-2 cells to a significantly greater extent than butyrate. It is also noteworthy that the SCFA combination treatment was observed to reduce GSH availability in Caco-2 cells to a greater extent than treatment with either SCFA alone (5mM) after 24hr but not after 48hr treatment.

Concomitant with the decreases in GSH availability by SCFA treatment observed within the current study, differential dose and time-dependent increases in OPP activity were detected in both Kato III and Caco-2 cells. Chapter 4 demonstrated the significant dose and time-dependent induction of OPP activity in Kato III cells by both butyrate and propionate. However, the induction of OPP activity was noted to be significantly greater (almost 10 fold) following butyrate treatment than propionate in this cell type.

Similarly, Chapter 7 described the up-regulation of OPP activity by SCFA treatment in Caco-2 cells. It was observed that short-term treatment (24hr) with the higher concentrations of butyrate and propionate (5mM and 10mM) led to significant increases in OPP activity. However, after longer-term treatment (48hr) only the highest concentration of butyrate (10mM) increased OPP activity in Caco-2 cells. Interestingly, the lowest concentration of butyrate (1mM) significantly reduced OPP activity after 48hr incubation while no effect was observed with 5mM butyrate treatment. In contrast, 48hr treatment with all concentrations of propionate led to significant increases in OPP activity with the lowest concentration of propionate (1mM) inducing significantly greater increases in OPP activity than 5mM or 10mM propionate. Hence, butyrate appeared to cause an acute up-regulation of OPP activity in Caco-2 cells, whereas the actions of propionate on OPP activity appeared to be more chronic. It should also be noted that after 48hr

incubation, the greatest increase in OPP activity of Caco-2 cells was over eight times less than the greatest increase induced by the SCFAs in Kato III cells.

These results are extremely interesting as they indicate that different SCFAs induce very different changes in OPP activity in Caco-2 and Kato III cancer cell lines. However, the demonstrated effects of both SCFAs on the OPP and GSH do not easily explain the overall cell death mechanisms also documented. However, it is proposed that changes to OPP activity and GSH availability within the two cell lines are associated with differential increases in the production of reactive oxygen species (ROS) by either SCFA.

ROS, including the hydroxyl radical ($^{\bullet}OH^{-}$) and the superoxide anion ($^{\bullet}O_2^{-}$), play an important role in the aetiology of many diseases of the gastrointestinal tract, including cancer [25, 27]. Under normal conditions, in the absence of SCFAs, the intracellular antioxidant defence system (OPP and GSH) is capable of protecting the intracellular milieu from increases in the production of ROS [91, 97]. However, some agents have the ability to attenuate this defence system and render it ineffective, such as dietary pectin [167] and specific inhibitors of GST (Nakajima *et al*., 2003). Indeed, butyrate has recently been demonstrated to induce apoptosis through the production of ROS [84, 85]. Therefore, any alterations to the antioxidant defence system following SCFA treatment would be aimed at reducing the impact of SCFA-induced ROS production. More specifically, it could be hypothesised that increased ROS production by SCFAs would induce increases in OPP activity and GSH availability.

In agreement with this hypothesis, butyrate and propionate were observed to induce up-regulation of the OPP in both Kato III and Caco-2 cells, although to different extents. This suggested that the SCFAs, particularly butyrate, caused the production of ROS and induced

changes to intracellular redox state in the cell lines studied. It is proposed that this led to the up-regulation of the OPP in order to reduce the oxidative state of the cell. However, GSH levels were reduced following SCFA treatment suggesting that the increased level of ROS production by SCFAs may have greatly outweighed the availability of GSH, and hence the capability of the antioxidant defence system. Ultimately, this resulted in a significant reduction in GSH availability and thus reduced the ability of the cancer cells to counteract further ROS-induced oxidative stress.

This thesis has also demonstrated that in Kato III cells, treatment with propionate led to a greater reduction in GSH than butyrate treatment. It is suggested that this phenomena is a result of the 10 fold lower OPP activity in Kato III cells following propionate treatment than butyrate treatment. This infers that the synthesis of GSH following butyrate treatment would be significantly greater than following propionate treatment. This has not been previously reported. Thus, it is proposed that differences in GSH availability between butyrate and propionate treated Kato III cells would be indicative of the rate of synthesis of GSH by the OPP. The current thesis did not directly measure the rate of GSH synthesis. Therefore, future studies are required to assess ROS production, GSH synthesis and GSH utilisation (oxidation) during SCFA treatment.

10.6: Modulation of the OPP and GSH availability by DHEA and DEM in Kato III cells

Chapter 5 described the potential for two inhibitors of intracellular protection to enhance the sensitivity of Kato III cells to butyrate and propionate-induced cell death. Kato III cells were incubated with dehydroepiandrosterone (DHEA), an inhibitor of the OPP, and diethyl maleate (DEM), a glutathione depleting agent, prior to the addition of either SCFA. It was hypothesised that DHEA and/or DEM would reduce GSH availability in this cell line leading to a reduction in

their protective ability against butyrate and propionate treatment, and in turn, leading to an increase in the induction of apoptosis.

It was demonstrated that both DHEA and DEM were capable of significantly reducing the viability of Kato III cells when used alone, and in combination with butyrate. However, the greatest reduction in cell viability was evident following butyrate treatment alone. In contrast, the pre-incubation of propionate-treated cells with DEM or DHEA led to significant reductions in cell viability, compared to propionate treatment alone. Therefore, the inhibition of intracellular protection by DEM and DHEA enhanced the sensitivity of Kato III cells to propionate, but not butyrate-induced apoptosis.

It is noteworthy, that although significant reductions in Kato III viability were demonstrated when propionate was combined with DHEA, compared to propionate treatment alone, this was significantly less than the reduction by DHEA treatment alone. These findings are extremely interesting as they suggest the lack of any additive effect on the induction of apoptosis when combining butyrate with DHEA. Potentially, the results of this study could be explained in two different ways: Firstly, it has been assumed that GSH is the primary antioxidant enzyme mediating the reduction of ROS within a cancer cell. This is not a complete oversight as many investigators have proposed this to be the case in a number of cancer cell types [31, 108, 168]. However, there are also many other important molecules involved in ROS scavenging, including the antioxidant enzymes superoxide dismutase (SOD) and catalase [101, 102]. In fact, previous studies have reported that $^{\bullet}O_2^-$ is sequentially detoxified by SOD to H_2O_2 and then into H_2O by either catalase or glutathione-peroxidase, a GSH-associated enzyme. This suggests that the maintenance of intracellular redox state is under far greater control than by GSH alone. Therefore, the loss of ROS scavenging ability by GSH may have been replaced by the

antioxidant activity of SOD and catalase. This suggests that inhibition of GSH, by DHEA or DEM at the concentrations tested within this thesis, was not sufficient to outweigh the total antioxidant activity of the Kato III cell.

The second explanation for these results comes from growing evidence supporting the role of ROS in the promotion of cell growth rather than cell death [97]. Recently, several investigators have demonstrated that low levels of ROS can actually cause the inhibition of apoptosis [95, 102]. This is extremely important when considering the potential of novel agents aimed at reducing resistance to current chemotherapeutics.

Pervaiz and Clement [97] described a number of studies implicating H_2O_2 as the main apoptosis-inducer, and opposingly, the role of $^{\bullet}O_2^-$ in the inhibition of apoptosis. Pervaiz *et al*. [102] reported that the inhibition of SOD reduced the induction of apoptosis in a melanoma cell line in response to anti-cancer drugs. Conversely, the overexpression of SOD in this cell line was demonstrated to enhance tumour cell sensitivity to drug treatment. This infers that the lack of $^{\bullet}O_2^-$ elimination by SOD inactivity leads to increases in intracellular $^{\bullet}O_2^-$ levels which are then able to reduce the induction of apoptosis. Moreover, the activity of caspase-3, the main apoptotic executioner, was reported to be suppressed by increased $^{\bullet}O_2^-$ levels and enhanced by reductions in its levels. Thus, within the current study, a reduction in GSH availability may have prevented the efficient reduction of $^{\bullet}O_2^-$, and hence reduced the activity of caspase-3. This would, in turn, prevent any additive induction of apoptosis during co-treatment with SCFAs and DHEA/DEM in the Kato III cells, as described.

Even more pertinent to the current thesis is the report that the $^{\bullet}O_2^-$ is a natural inhibitor of Fas-mediated cell death [95]. Within this thesis, we have hypothesised that butyrate and propionate-induced cell death may be Fas-mediated in Kato III cells. This is due to the fact that Kato III cells are p53-deficient and thus must undergo apoptosis in a p53-independent manner. Therefore, we propose that an inhibition of Fas-mediated cell death may have caused the lack of any significant increase in apoptosis observed when Kato III cells were co-incubated with SCFAs and DHEA/DEM. Further studies are required to properly determine the relationship between ROS levels, Fas inhibition and cell death in response to SCFAs, DHEA and DEM in this Kato III cell line.

10.7: Stable isotope-based assessment of D-glucose and butyrate metabolism in Kato III and Caco-2 cells

Very few studies have investigated the effects of SCFAs on glucose metabolism. However, a recent study demonstrated that the metabolism of D-glucose was significantly reduced after 72hr butyrate treatment in the colon cancer cell line, HT29 [38]. In contrast, Chapter 4 and Chapter 7 demonstrated increases in D-glucose metabolism after 24hr and 48hr incubation with all SCFA treatments in both Kato III and Caco-2 cells. However, after 48hr incubation, differences in D-glucose metabolism became evident. For example, a downward trend in D-glucose metabolism by butyrate and combination SCFA treatment, and an upward trend by propionate treatment were observed from 24hr to 48hr in both cell lines. Interestingly, 72hr incubation of Kato III cells with butyrate reduced D-glucose metabolism while propionate increased its metabolism. Potentially, this inferred that an incubation period of greater than 48hr is required before butyrate is preferentially utilised for energy production over D-glucose in both Caco-2 and Kato III cancer cell lines. Additionally, in contrast to butyrate, these results suggest that propionate is not utilised as an energy source within either cell line, and this has not previously been documented.

Chapter 8 investigated the capability of Kato III and Caco-2 cells to metabolise butyrate due to their demonstrated differences in sensitivity to this SCFA. Previous studies proposed that differences in the metabolism of butyrate may regulate the sensitivity of different cell lines to butyrate [38, 70]. It was, therefore, hypothesised that differences in the apoptosis-inducing effect of butyrate may be due to differences in the rates of butyrate metabolism between the Kato III and Caco-2 cell lines.

In agreement with this hypothesis, it was observed that Caco-2 cells metabolised butyrate at a significantly greater rate than Kato III cells. This supports the findings of Boren *et al*. [38], who demonstrated that the sensitivity of a cell to butyrate was mediated by its ability to metabolise this SCFA. Moreover, these results suggest that the rate of butyrate metabolism is directly related its ability to induce apoptosis. This also suggests that products of butyrate metabolism are the main mediators of its effects on cell viability, rather than butyrate alone. Indeed, Leschelle *et al*. [71] proposed that the action of butyrate is most likely mediated through the generation of metabolites by mitochondria, such as β-hydroxybutyrate.

Chapter 8 also demonstrated significant differences in the profiles of butyrate metabolism between Kato III and Caco-2 cells. Specifically, the metabolism of butyrate was demonstrated to begin almost immediately (≤15min) after the addition of 1-^{13}C-butyrate to Kato III cells. In contrast, the metabolism of 1-^{13}C-butyrate by Caco-2 cells began after 45min. Thereafter, a linear rate of $^{13}CO_2$ production was achieved in Kato III cells after 30min but not until 60min in Caco-2 cells.

The profile of butyrate metabolism has not previously been documented within any cell type, mainly due to the lack of an available technique. However, the results of the current study are in

agreement with a recent report demonstrating a significant reduction in expression of the MCT1 butyrate transporter in colon cancer cells [79]. It is proposed, therefore, that differences in the profiles of butyrate metabolism described in the current study are due to differences in butyrate transport across the cell membrane, between Kato III and Caco-2 cells.

10.8: Differential gene expression patterns in AGS (gastric cancer) and Caco-2 cells following butyrate treatment

Chapter 9 described a pilot study undertaken to assess the expression of genes that were hypothesised to be altered by treatment with butyrate. This was undertaken in an AGS gastric adenocarcinoma cell line and the Caco-2 colon carcinoma cell line. Equivalent reductions in the viability of both AGS and Caco-2 cells by butyrate treatment after 24hr (down by 62%) and 48hr (down by 97%) incubation, were initially demonstrated. This is in contrast with the reduced effect of butyrate on Kato III cells that showed maximal reductions in viability of only 14% (after 72hr). It is proposed, however, that this was due to a functional p53 gene status of AGS cells [169] compared to Kato III cells, which are p53-null.

The genes assessed within this pilot study included: Bcl-2 (anti-apoptotic), Bcl-X_L (antiapoptotic), Bax (proapoptotic), ODC (cell proliferation marker), $p21^{Waf1/Cip}$ (cell cycle regulator), Cdx-2 (involved in development), Hes-1 (involved in differentiation) and Msi-1 (proposed stem cell marker). The most interesting finding was the up-regulated expression of Bcl-2 and Bcl-X_L in AGS cells, compared to their down-regulation in Caco-2 cells. Furthermore, Bax expression was down-regulated in both AGS and Caco-2 cells, although to a greater extent in the latter.

Recent studies have demonstrated a reduction in Bcl-2 expression and an increase in Bax expression by butyrate in both a gastric cancer cell line and a lymphoma cell line [41, 44]. The opposing expression levels of Bcl-2 and Bax demonstrated within AGS cells suggest that this SCFA exerts differential effects on the regulation of gene expression in different cell lines. Butyrate is known to mechanistically alter gene expression by inducing histone hyperacetylation through inhibition of histone deacetylase [79]. Thus, differences may exist in the degree of histone hyperacetylation by butyrate between different cell lines that may explain differences in the expression of a variety of genes by this SCFA.

10.9: Final conclusions and potential significance

Firstly, this thesis has described a novel method for the rapid assessment of cellular metabolism that could be easily applied to many other substrates and cell types. This technique was utilised to measure alterations to glucose and butyrate metabolism by SCFAs within a gastric cancer and a colon cancer cell line. Secondly, and most importantly, this thesis has demonstrated that SCFAs, particularly butyrate, have the capability of inducing apoptosis, reducing viability and inducing G2-M arrest in gastric and colon cancer cells. However, in contrast to the original hypothesis, Caco-2 colon cancer cells were demonstrated to be significantly more sensitive to SCFA-induced apoptosis than Kato III gastric cancer cells. Thirdly, this thesis has demonstrated that the sensitivity of a particular cancer cell type to butyrate is directly related to its ability to metabolise this SCFA. Fourthly, this thesis has proposed that the sensitivity of a cancer cell to SCFA treatment is dependent on their functional p53 status. Fifthly, this thesis demonstrated that SCFA-induced cell death was partly mediated by a reduction in intracellular protection. And finally, this thesis has reported the differential expression patterns of a number of apoptosis and differentiation-associated genes by butyrate, in AGS gastric cancer cells and Caco-2 colon cancer cells.

The results of this thesis suggest that mechanistically, the response of cancer cells to SCFAs is complex, and involves multiple distinct mechanisms and pathways. It appears that the sensitivity of a cancer cell to SCFAs, particularly butyrate, is mediated by a number of factors, including the ability of these agents to activate p53 and Fas, leading to the induction of cell cycle arrest and apoptosis (Figure 10.3). This is concomitant to the induction of oxidative stress, and hence changes to intracellular redox state that appear to enhance the apoptotic effects of these SCFAs. However, in cell lines without functional p53 status, such as Kato III, it appears that the induction of apoptosis by SCFAs is mediated via p53-independent pathways which do not appear to induce apoptosis to the extent seen within cell types containing functional p53, such as Caco-2 (Figure 10.4).

In conclusion, this thesis has provided evidence that SCFAs, particularly butyrate, can induce apoptosis and cell cycle arrest in gastric cancer cells and colon cancer cells *in vitro*. It is proposed that butyrate, or a combination of both SCFAs, has the potential to be utilised as an adjunctive therapy for the treatment of both gastric cancer and colon cancer. The further identification of additional effectors of SCFA-induced apoptosis, and a better understanding of the underlying mechanisms will enhance the future development of more effective agents to treat gastric and colon cancer. Future studies should combine butyrate with current and novel chemotherapeutic agents to potentially enhance the sensitivity of gastric and colon cancer cells to the induction of apoptosis. Hence, further investigations into the capability of this SCFA to increase chemotherapeutic efficacy are required.

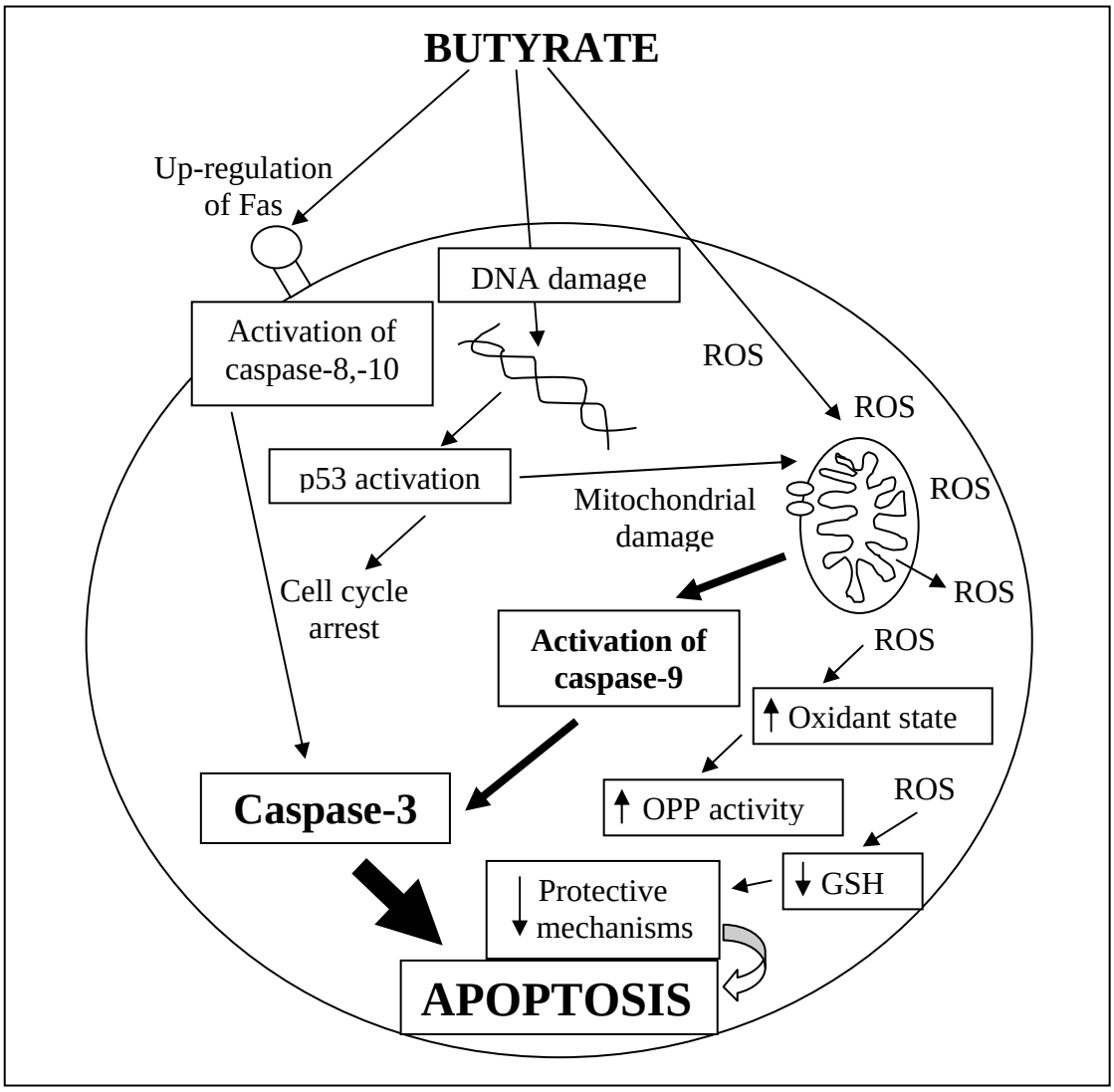

Figure 10.3: Schematic diagram depicting the proposed pathways of apoptosis induction in the Caco-2 colon cancer cell line. It is proposed that butyrate treatment induces the activation of the extrinsic Fas pathway while concomitantly activating the intrinsic p53 and ROS-mediated pathways of cell death. The production of ROS, potentially by the metabolism of butyrate, leads to an increase in the oxidative state of the cell and the subsequent induction of the OPP. It is proposed, however, that the ROS load outweighs the rate of GSH synthesis. This leads to a reduction in GSH and hence, intracellular protection. Ultimately, these mechanisms culminate in the induction of apoptosis.

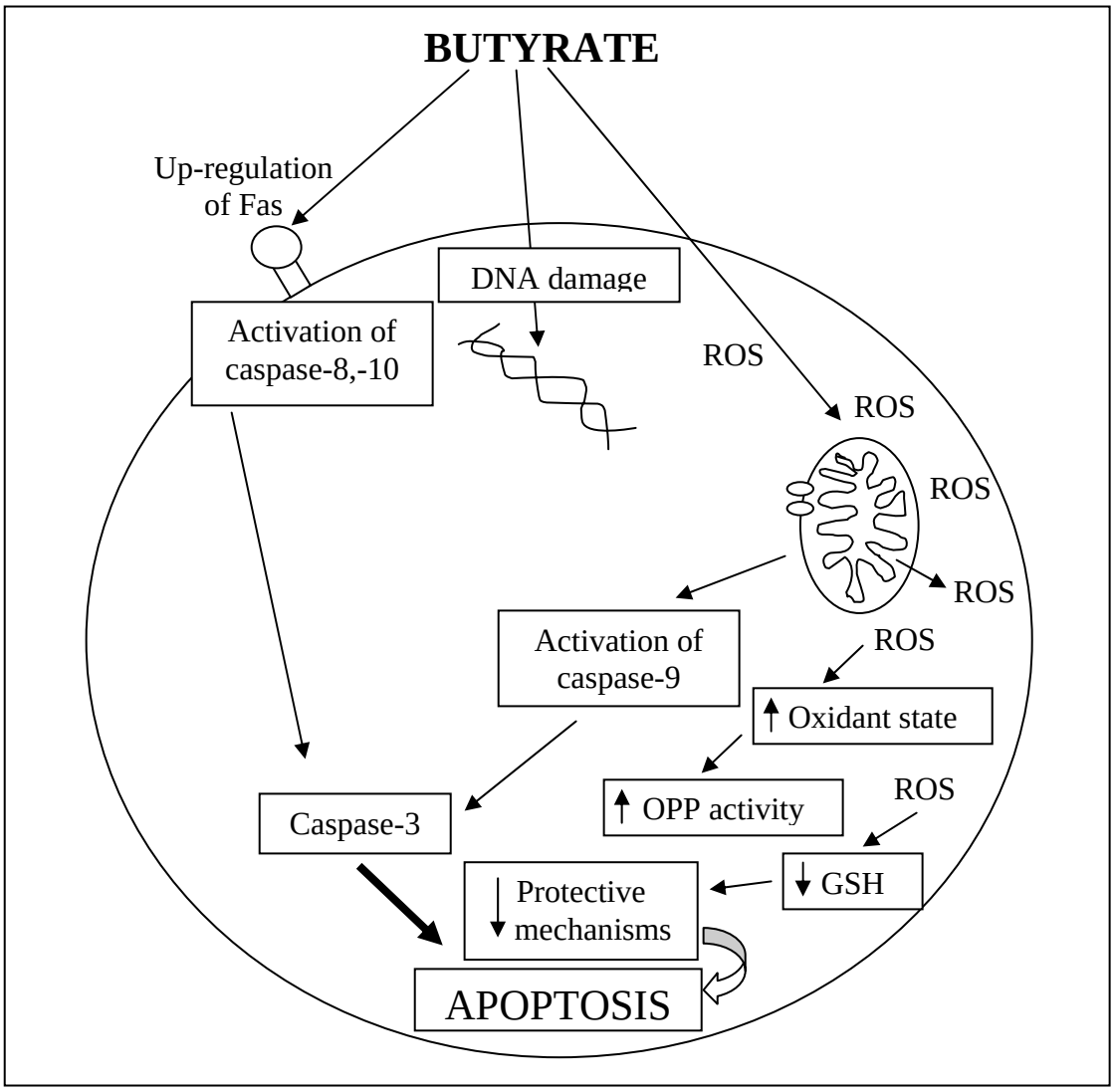

Figure 10.4: Schematic diagram depicting the proposed pathways of apoptosis induction in the Kato III gastric cancer cell line. It is proposed that butyrate induces the activation of the extrinsic Fas pathway while also activating the ROS-mediated (p53-independent) pathway of cell death, although to a much lesser extent than in Caco-2 cells. ROS production leads to an increase in the oxidative state of the cell and the induction of the OPP. Again, ROS load outweighs the rate of GSH synthesis. This leads to a reduction in GSH and hence, intracellular protection. Ultimately, these mechanisms culminate in the induction of apoptosis, but to a much lesser extent than that seen in Caco-2 cells.

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APPENDIX A: Publications arising from this thesis

Papers:

Geoffrey M Matthews, GS Howarth and RN Butler. Nutrient and antioxidant modulation of apoptosis in gastric and colon cancer cells (Review). *Cancer Biology and Therapy,* April 2006. 5(6): 569-572

Geoffrey M Matthews, GS Howarth and RN Butler. Short-chain fatty acid modulation of apoptosis in the Kato III gastric cancer cell line. *Manuscript accepted for publication in Cancer Biology and Therapy, January 2007.*

Geoffrey M Matthews, Gordon S Howarth and Ross N Butler. D-glucose metabolism in the Kato III gastric cancer cell line. *Manuscript in preparation.*

Geoffrey M Matthews, GS Howarth and RN Butler. Short-chain fatty acid modulation of apoptosis in the Caco-2 colon cancer cell line. *Manuscript in preparation.*

Geoffrey M Matthews, GS Howarth and RN Butler. A stable isotope-based profile of butyrate metabolism in Kato III and Caco-2 cell lines. *Manuscript in preparation.*

Geoffrey M Matthews, GS Howarth and RN Butler. Inhibition of intracellular protection by diethyl maleate and dehydroepiandrosterone enhances the sensitivity of Kato III cells to short-chain fatty acid-induced apoptosis. *Manuscript in preparation.*

Abstracts:

Geoffrey M Matthews, Gordon S Howarth and Ross N Butler. Inhibition of intracellular protection enhances the sensitivity of Kato III gastric cancer cells to SCFA-induced apoptosis. *Poster presented at the Lorne Cancer Conference, Victoria, Australia*, Feb 2007.

Geoffrey M Matthews, Gordon S Howarth and Ross N Butler. Inhibition of intracellular protection enhances the sensitivity of Kato III gastric cancer cells to SCFA-induced apoptosis. *Submitted for presentation at the Digestive Disease Week conference, USA,* May 2007.

Geoffrey M Matthews, Gordon S Howarth and Ross N Butler. Short-chain fatty acid modulation of apoptosis and cell cycle in Kato III gastric cancer cells. *4th International Gastrointestinal Carcinogenesis conference proceedings, August 2006.*

Geoffrey M Matthews, Gordon S Howarth and Ross N Butler. Short-chain fatty acid modulation of cell cycle in Kato III gastric cancer cells. *Journal of Gastroenterology and Hepatology 2006: 21(Suppl 4): A360*

Geoffrey M Matthews, James W Wilson, Gordon S Howarth and Ross N Butler. Up-regulation of intracellular protective mechanisms in gastric cancer cells following exposure to sodium butyrate. *Australian Society for Medical Research, Adelaide, June 2006.*

Geoffrey M Matthews, James W Wilson, Gordon S Howarth and Ross N Butler. Up-regulation of intracellular protective mechanisms in gastric cancer cells following exposure to sodium butyrate. *Lorne Cancer Conference proceedings, February 2006.*

Laurens Kruidenier, Saif Khair, **Geoffrey Matthews**, Ian Sanderson and James Wilson. The expression of the stem cell marker, *Musashi-1*, in Caco2 cells following butyrate treatment and during differentiation. *Institute of Cell and Molecular Science symposium, Centre for Adult and Paediatric Gastroenterology, Barts and The London, October 2005.*

Geoffrey M Matthews, Gordon S Howarth and Ross N Butler. Butyrate and propionate differentially induce apoptosis in Kato III gastric cancer cells. *Journal of Gastroenterology and Hepatology* 2005; 20(suppl 1): A109

Geoffrey M Matthews, Gordon S Howarth and Ross N Butler. Butyrate and propionate significantly increase the activity of the oxidative pentose pathway in Kato III gastric cancer cells. *Journal of Gastroenterology and Hepatology* 2005; 20(suppl 1):A109

Geoffrey M Matthews, Gordon S Howarth and Ross N Butler. Butyrate and propionate differentially induce apoptosis in Kato III gastric cancer cells. *Abstract presented at the World Congress of Gastroenterology, Montreal, Canada,* 2005.

Geoffrey M Matthews, Gordon S Howarth and Ross N Butler. Butyrate and propionate significantly increase the activity of the oxidative pentose pathway in Kato III gastric cancer cells. *Abstract presented at the World Congress of Gastroenterology, Montreal, Canada,* 2005.

GM Matthews, GS Howarth and RN Butler. Monitoring shifts in metabolic flux by assessment of 1-^{13}C-D-glucose oxidation and oxidative pentose pathway activity in a gastric cancer cell line. *Proceedings of the Digestive Disease Week conference in Chicago, May 2005.*

GM Matthews, GS Howarth and RN Butler. Metabolism of 1-^{13}C-D-glucose in the Kato III gastric cancer cell line. *Proceedings of the 2nd Australian Health and Medical Research Congress, Sydney, Australia*, 2004.