NOTE: All figures have been placed at the end of the thesis.

1 Introduction

Morbidity and mortality resulting from colorectal cancer (CRC) is of major concern, particularly in developed countries of the world. There are many factors that contribute to the development and progression of CRC, and dietary and genetic aspects are thought to be of particular importance. There is much evidence to suggest that increased fibre intake is associated with a decreased risk of CRC and a number of mechanisms are thought to contribute to this effect. This introduction will summarise the existing information available relating to CRC development and prevention, with particular emphasis on the proposed mechanisms of action of fibre, and more specifically the mode of action of butyrate (BuA), a fermentation product of fibre.

1.1 Development and progression of CRC

1.1.1 Normal colon physiology

Both the small and large intestine have a single layer of columnar epithelium cells, which are used to transfer nutrients and fluid to the circulation and maintain a barrier against toxins and infectious agents (Johnson, 2002). All cells in the colonic epithelium arise from rapidly proliferating stem cells located in the crypts. Migration of the cells occurs from the base of the crypt up to the luminal surface (Potten and Morris, 1988). As this migration occurs the cells undergo differentiation and mature cells near the luminal surface undergo programmed cell death, defined as apoptosis, and are shed into the gut lumen (Figure 1.1) (Kaur and Potten, 1986). Proliferation in normal colonic tissue occurs in the basal 40% of the crypts, with the stem cell cycle time taking approximately 36 hours, and migration estimated to take 3-8 days (Lipkin et al., 1963).

1.1.2 Disruptions to normal colonic physiology can increase risk of CRC

The processes of cell proliferation, differentiation, migration and apoptosis are highly co-ordinated (Mariadason et al., 2002), with a dynamic equilibrium required for maintenance of colonic homeostasis (Potten et al., 1997) (Hall et al., 1994). Disruption to the delicate equilibrium leading to increased cellular growth can cause neoplasia (Michor et al., 2005) and increase the risk of developing CRC (Bedi et al., 1995) (Comalada et al., 2006).

CRC is thought to develop through an adenoma-carcinoma pathway (Hill, 2000) with morphological changes in this pathway associated with progressive mutations (Figure

1.2) (Rajagopalan et al., 2003) (Vogelstein et al., 1988). These mutations occur in genes that control cell growth and differentiation (Vogelstein et al., 1988) (Johnson, 2002) with each mutation thought to produce defective cell regulation leading to a growth advantage. A series of cell mutations is thought to cause the conversion of normal cells into cancerous cells (Ilyas et al., 1999). The model of acquired mutations implies that cancer arising from genetic instability usually takes 20-40 years to develop (Rajagopalan et al., 2003) and requires a number of factors to drive progression towards malignancy (Michor et al., 2005).

1.1.3 Common mutations associated with CRC

Mutations in the Adenomatous polyposis coli (APC) gene are often considered the first step or "gatekeeper" in CRC development (Lamlum et al., 2000) (Powell et al., 1992) (Ilyas et al., 1999), with 60-80% of sporadic CRCs found to express mutant APC (Bodmer et al., 1987) (Cottrell et al., 1992) (Nakamura, 1993). APC plays an important role in the regulation of the *wnt*/β-catenin signal transduction pathway which is responsible for the transcription of the oncogene c-myc (Morin et al., 1997) (He et al., 1998). Mutations in APC and β-catenin have been shown to have the same physiological outcome (Sparks et al., 1998). In cancers lacking APC mutations, β-catenin is mutated in approximately 50% of cases (Sparks et al., 1998) indicating that almost all sporadic CRCs have a mutation in this signal transduction pathway via mutations to either APC or β-catenin. Cells in the crypt that are APC or β-catenin mutant become dysplastic and almost invariably develop into small polyps (Rajagopalan et al., 2003).

Colorectal polyps are fleshy growths that protrude from the lining of the colon that are usually benign and are often referred to as adenomas. Small adenomas rarely develop to malignancy with adenomas smaller than 3mm in diameter being classed as having very low malignancy risk. However, a small proportion of adenomas grow to a large size and adenomas which are 2cm or above often remain benign, but are mildly dysplastic and much more likely to develop into carcinoma (Morson, 1974). Consequently, the growth of an adenoma is seen as a key step in the development of carcinomas (Hill, 2000). Rajagopalan et al (Rajagopalan et al., 2003) suggest that other mutations which allow the growth of the small lesion into a large polyp or adenocarcinoma occur as a part of the disease progression.

Other commonly reported gene mutations thought to be involved in CRC disease progression include K-RAS and p53. Mutations in the p53 gene are found in up to 70% of sporadic CRCs and are correlated with the transition from benign adenoma to malignant carcinoma (Ilyas et al., 1999). The role of the p53 gene is described in detail in the review by Vogelstein and Kinzler (Vogelstein and Kinzler, 1992). The key findings from this report indicate that p53 is a tumour suppressor gene and is central to many apoptosis and cell cycle pathways. Therefore, mutations in this gene are likely to lead to deregulated cell growth. The RAS family of proteins are involved in signal transduction pathways in almost all cell types, with mutations of K-RAS shown to affect cell adhesion and cell cycle progression (Wong et al., 1998) (Yang et al., 1998) (Ilyas et al., 1999). Mutations in the K-RAS gene are observed in approximately 10–20% of small adenomas and 40–50% of larger adenomas and carcinomas, which suggests this alteration is an important event in the progression of adenoma to carcinoma (Fearon and Vogelstein, 1990).

Genomic instability that leads to growth or survival advantages is also considered a key event in the progression of CRC (Young et al., 2005). Multiple gene dysfunction leads to genetic instability, which becomes increasingly disordered with the progression to malignancy (Young et al., 2005). Two different types of genetic instability have been reported in CRC (Michor et al., 2005) (Rajagopalan et al., 2003). Approximately 15% of all sporadic CRCs have loss in mismatch-repair gene expression which lead to microsatellite instability (MIN) at the nucleotide level (Rajagopalan et al., 2003). In the remaining 85% of sporadic CRC chromosomal instability is present, which leads to a loss or gain of the whole chromosome or parts of chromosomes during cell division (Rajagopalan et al., 2003) (Cahill et al., 1998).

There are also a number of other non-somatic mutations that are involved in the progression of CRC (Johnson and Lund, 2007). These are thought to be driven by epigenetic modifications, such as gene silencing via methylation of the promoter regions of tumour suppressor genes (Baylin et al., 1998). Although the mechanism does not involve modification of the DNA sequence, the changes can still be passed on through mitosis (Esteller and Herman, 2002) (Feinberg, 2001). The reports highlighted in this section show that the development of CRC is a complex, multistep process and many gene mutations and epigenetic events associated with CRC progression exist. A comprehensive review of candidate genes involved in the development and progression of CRC can be found in Ilyas et al (Ilyas et al., 1999).

1.2 Factors influencing the development of CRC

CRC is a major problem in the developed countries. Reports suggest that approximately 5-15% of CRC is caused in dominantly inherited patterns (Cannon-Albright et al., 1988). However epidemiological data has shown that there is a major geographical variation in the incidence of CRC (Parkin et al., 2005). This indicates that although some CRC is hereditary, environmental factors play a large role in the development of this type of cancer. The genetic factors associated with familial CRC and the environmental components which modulate the risk of developing this disease will be described in the next section.

1.2.1 Inherited genetic risk factors

The two major and best defined forms of familial CRC are Familial Adenomatous Polyposis (FAP) and Hereditary Nonpolyposis Colorectal Cancer (HNPCC) (Kinzler and Vogelstein, 1996). FAP is an inherited dominant autosomal disease affecting approximately 1 in 7000 people (Lynch et al., 1996). Patients with FAP have a mutated APC gene and consequently develop hundreds to thousands of small polyps or adenomas within their first 30 years of life (Kinzler and Vogelstein, 1996) leading to cancer by the age of 40-50 (Potter, 1999) (Lynch and de la Chapelle, 2003). This is approximately 20 years earlier than people not affected by FAP (Potter, 1999). As previously described, it is rare for polyps to develop into invasive carcinomas and although the polyps of FAP patients are benign, the vast number of lesions dramatically increases the risk of carcinoma development (Kinzler and Vogelstein, 1996) (Byers et al., 1997).

HNPCC patients also have a higher risk of developing CRC, however the increased risk is observed in the absence of large numbers of adenomatous polyps (Calvert and Frucht, 2002). Like FAP, HPNCC is an inherited autosomal dominant disease, however, patients with this condition have mutations in one of several DNA mismatch repair genes which accelerate the progression of tumour development (Lynch et al., 1996).

Other genetic factors have also been demonstrated to influence the age of onset of colorectal cancer, with an average 10 year earlier onset of CRC in people who have a first-degree relative with this disease (Fuchs et al., 1994). More details relating to the hereditary causes of CRC can be found in the review by Ilyas et al (Ilyas et al., 1999) or Potter (Potter, 1999).

1.2.2 Environmental risk factors

Although CRC is the 2nd most commonly diagnosed cancer in the Western World, the majority of CRC incidence is sporadic (Lynch and de la Chapelle, 2003). Therefore, the onset of disease is considered to be highly influenced by environmental factors (Wargovich et al., 1988) (Reddy et al., 1989) (Potter et al., 1993) (Potter, 1996). Environmental risk factors are thought to include obesity (Gunter and Leitzmann, 2006) (Kune et al., 1990), high meat intake (Lipkin et al., 1999) (Norat et al., 2005) and alcohol consumption (Potter, 1999) and smoking (Giovannucci et al., 1994a) (Giovannucci et al., 1994b) (Knekt et al., 1998). However, an inverse relationship between CRC and non-steroidal anti-inflammatory drugs (NSAIDS) (Lancaster and Silagy, 1994) (Potter, 1999) fish and/or omega 3 consumption (Norat et al., 2005) (Willett et al., 1990) and physical activity (Potter et al., 1993) have been demonstrated. By far one of the largest environmental risk factors for developing CRC is diet and there are now a number of epidemiological studies that have linked diet, and more specifically fibre, with a lowered risk of CRC. The evidence to support the epidemiological data which shows a decreased risk of CRC with a high fibre diet is presented in the following section. As the main focus of this report is the relationship between diet and CRC the risk factors of physical activity, body mass and smoking have not been discussed in detail. For a comprehensive review of the other environmental risk factors please refer to the publication by Potter (Potter, 1999), Gunter and Leitzmann (Gunter and Leitzmann, 2006) or Johnson and Lund (Johnson and Lund, 2007).

1.3 Diet as a risk factor for CRC

It is now considered that approximately 60-80% of CRC in Western countries is attributed in some way to diet (Bingham, 2000) (Chapkin et al., 2000). In less developed countries the age adjusted incidence rate of CRC is approximately 20% less than that of Western countries (Johnson and Lund, 2007). Australia and New Zealand have one of the highest CRC incidence rates of the world, with 50 cases per 100,000 people compared to the lowest rate in Bangladesh of 1 per 100,000 (Parkin et al., 2005). Studies have shown that this is not an inherent difference in the genetic make up of individuals in each country as migrants who have moved from a country with a low CRC incidence rate to an area of high incidence assume the risk of the new population (Flood et al., 2000). Similarly, countries that have seen a rise in affluence have also reported a rise in the incidence of CRC (Johnson and Lund, 2007). Japan provides an example of this trend, as it traditionally had low CRC rates, but has recently undergone

Westernisation and has observed a steady increase in CRC incidence. The rate of CRC incidence has increased so dramatically that Japan now has one of the highest age adjusted incidence rates in the world (Key et al., 2002).

One of the first reports to link diet to an altered risk of CRC was published in 1971 by Burkitt (Burkitt, 1971). In this paper the author proposed a possible link between bowel cancer and intake of fibre, which led to the "dietary fibre hypothesis". In light of this hypothesis, in 1986 Trowell and Burkitt (Trowell and Burkitt, 1986) proposed that the geographical variation in CRC incidence could be attributed to the consumption of very high levels of non-digestible polysaccharides derived from unrefined plant cell walls in countries found in Asia and Africa compared with low levels of fibre intake in Western countries.

1.4 Fibre as a risk factor for CRC

Fibre is sourced from a number of different plant foods and is mainly found in vegetables, fruit, grains, seeds, nuts and legumes (Kim, 2000). Fibre is heterogeneous in nature (McIntyre et al., 1993) and predominantly consists of non-starch polysaccharides and nonpolysaccharides (Harris and Ferguson, 1993). Nonpolysaccharides mostly consist of lignins and non-starch polysaccharides include celluloses, hemicelluloses, pectin, gums and mucilages (Kim, 2000) (Young et al., 2005).

In the early 1980s, Stephen et al (Stephen et al., 1983) demonstrated that most starch was digested early in the gastrointestinal tract and only 5-10% of dietary starch reaches the colon intact. Consequently, fibre is now broadly categorised into 2 major classifications: soluble and insoluble. Soluble fibre is highly fermentable in the proximal colon while insoluble fibre is slowly fermented and reaches the distal colon undigested (Harris and Ferguson, 1993) (Young et al., 2005). Although there is currently no internationally accepted definition of "dietary fibre" (Wasan and Goodlad, 1996) (Young et al., 2005) (Kim, 2000), Young et al (Young et al., 2005) describe dietary fibre "in simple terms as complex carbohydrates that reach the colon".

1.4.1 Human trials investigating fibre and CRC risk

Since the "dietary fibre hypothesis" was first proposed by Burkitt, a number of human studies have been undertaken to further understand this relationship (Potter, 1999)

(Johnson and Lund, 2007) (Young et al., 2005). Many epidemiological, case controlled, prospective and intervention studies have been conducted, with a number of authors later conducting meta-analyses on the pooled data. There is an abundance of literature published in this area which has been well described in the following review papers; Potter (Potter, 1999) Young et al (Young et al., 2005) Kim (Kim, 2000) and Johnson and Lund (Johnson and Lund, 2007). An overview of the evidence for the protective effects of fibre is presented in this Chapter.

1.4.1.1 Correlation studies

Correlation studies examine the relationship between the per capita prevalence, incidence, or mortality rate of CRC in the population with dietary factors. Kim (Kim, 2000) conducted a comprehensive review of the epidemiological studies published between 1970 and 1999. In this report, 28 published international, within-country correlation and time-trend studies of CRC, fibre, vegetables, grains, fruits and cereals were reviewed. The authors describe that of the 28 studies, 23 (82%) show a strong or moderate protective effect of "fibre rich foods" on CRC development. Of the remaining studies, 4 showed no protective effects of fibre rich foods and 1 showed an increased risk. These results provide strong evidence to support the dietary fibre hypothesis, and these findings are also supported in the reviews by Johnson and Lund (Johnson and Lund, 2007) and Potter (Potter, 1999).

1.4.1.2 Case controlled studies

Case controlled studies compare the dietary patterns of individuals with CRC against the diets of people without CRC (Kim, 2000) (Obrador, 2006). In 1990, Trock et al (Trock et al., 1990) reported an odds ratio for CRC of 0.57 for the highest quartile of fibre intake compared with the lowest quartile following meta-analysis of 16 case controlled studies. Two years later, Howe et al (Howe et al., 1992) reported a similar odds ratio of 0.53 in the highest quartile of fibre consumption compared with the lowest quartile after conducting meta-analysis of 13 case control studies. The quartiles corresponded to fibre intakes of greater than 31g of fibre per day compared with less than 10g in each group respectively.

Friedenreich et al (Friedenreich et al., 1994) comprehensively reviewed 13 case controlled studies and reached a similar conclusion as the 2 reports described above. In this report, subjects who consumed greater than 27g of fibre per day had a 50%

reduction in CRC risk compared with subjects consuming less than 11g of fibre per day. Young et al (Young et al., 2005) presented a summary of case controlled studies that have investigated the evidence for protective effects of fibre in humans. This group concluded that in the majority of case controlled studies a strong or moderate protective effect of diets high in fibre exists, with an approximate 50% reduction in CRC risk with dietary consumption of approximately 30g of fibre per day.

1.4.1.3 Intervention trials

As the name suggests, intervention studies modulate the diet of individuals in the population and compares them with others that have continued with their "normal" diet. Human intervention studies should provide conclusive evidence to support or disprove a hypothesis, however, they are often difficult to conduct as large numbers of subjects are required to obtain statistical significance and the trials must continue for many years as CRC develops and progresses slowly (Kim, 2000). Strategies to overcome these issues have been developed, which include the investigation of dietary effects on individuals with a high risk of CRC or using intermediate biomarkers of CRC such as adenoma, proliferation markers, mitotic index, DNA aneuploidy aberrant crypts, mucins and a number of molecular biomarkers (Einspahr et al., 1997).

A number of intervention trials have been conducted and these have been reviewed in detail by Kim (Kim, 2000), with no conclusive evidence to support a preventive role of fibre and CRC. Several major weaknesses were, however, identified including short follow-up, small subject numbers, poor compliance with dietary intervention and high dropout rates. Furthermore, the use of intermediate biomarkers has not been proven to be associated with a decreased risk of CRC (Einspahr et al., 1997). It is also difficult to make definitive conclusions about the effect of diet if the subject populations are high risk individuals, such as those with genetic predispositions, as these people may already have precancerous lesions (Kim, 2000). The final conclusion regarding intervention trials by Kim (Kim, 2000) was that the results should be interpreted with caution, and should not be considered to refute the results from other more conclusive study types; such as correlation and epidemiological studies. Similar conclusions were also made by Young et al (Young et al., 2005).

1.4.1.4 Prospective and cohort trials

Prospective studies follow the diet of a large population over time and assess this against the incidence of disease. The report by Kim (Kim, 2000) reviewed 10 prospective studies and demonstrated that only 5 show a decreased risk of CRC with high fibre consumption. Similarly, Bingham et al (Bingham et al., 2003) reported that large prospective studies investigating the relationship between fibre and decreased risk of developing CRC and adenomatous polyps have not conclusively demonstrated a protective role of fibre. However, in prospective studies a number of limitations exist in the experimental procedures. Recruited subjects are generally only required to complete food frequency questionnaires at the commencement of the project (Kim, 2000). This assumes that their dietary intake did not change over the follow-up time of the study, which was typically 2-8 years (Kim, 2000). Additionally the follow up time in most prospective studies was also considered to be insufficient to observe significant effects as CRC is thought to progress over 20-40 years (Rajagopalan et al., 2003) (Young et al., 2005). Therefore, from prospective studies it appears that the evidence for a protective role of fibre has not been confirmed (Young et al., 2005).

One of the largest and most recent prospective cohort studies conducted is the European Prospective Investigation in Cancer and Nutrition (EPIC). This study was initiated in 1992, and monitors 519, 978 men and women from 10 European countries (Bingham and Riboli, 2004) and was designed to overcome some of the limitations associated with other prospective studies. The populations studied are from different countries with diverse dietary practices, making differences between dietary components easier to determine (Potter, 2003). Furthermore, food consumption in the EPIC study is measured by country specific questionnaires which captured local dietary practices and has improved compliance. Unlike other prospective studies, the EPIC study measured a much greater variety of variables for each patient. Personal lifestyle data and physical measurements are recorded along with each subject's history of tobacco and alcohol consumption, physical activity education, socio-economic status and employment history. Additionally, biological samples, including plasma, serum, leukocytes and erythrocytes have been collected and stored for over 400,000 of the participants. This study has provided, and will continue to provide precise epidemiological data relating to the effects of diet on different types of cancer, which can now be accurately matched to molecular factors via the testing of stored biological samples.

Five years after the commencement of the EPIC study, Bingham et al (Bingham et al., 2003) reported that 1,056 of the approximately 521,000 recruited subjects had developed CRC, and demonstrated that dietary fibre intake was significantly inversely correlated to the risk of CRC. This report demonstrated a relative risk of 0.58 for the highest versus lowest quintile of fibre intake from food. In a review by the same authors in 2004 (Bingham and Riboli, 2004), this finding was correlated to a recommendation that a doubling of fibre intake above the average levels consumed in most populations in the EPIC study (20g/day) could halve the risk of CRC.

Despite the many studies that have investigated the relationship of fibre to CRC, the data is not always consistent and some reviews have been sceptical of the effects of fibre (Obrador, 2006). Wasan et al (Wasan and Goodlad, 1996) acknowledge that there are a number of studies which link fibre to decreased CRC risk, however, also note that it is difficult to determine if a single class or constituent nutrient can be attributed to the decreased CRC risk when broad based changes to whole foods, such as fruits and vegetables, have been conducted in the majority of human trials. This is especially true when considering fibre supplementation trials in humans that have failed to show any significant effects (Wasan and Goodlad, 1996).

1.4.2 The type of fibre is important when investigating CRC risk

In the studies described in this section, most have not tried to differentiate between fibre sources (Young et al., 2005), and are based on measurements of “crude fibre”. Since fibre is heterogeneous in nature, it is possible that the moderate or statistically insignificant effects of fibre protection may have been more pronounced if the “type” of fibre was controlled for. Freudenheim et al (Freudenheim et al., 1990) recognised that different fibre types may affect the colon differently and investigated different fibre sources and the risk of CRC. These researchers categorised foods into either fruit/vegetable or grain groups. Each group was then further categorised into soluble or insoluble fibres and again into one final category consisting of the components of insoluble fibre; hemicellulose, cellulose and lignin (Figure 1.3). The results from this study indicate a decreased risk of CRC with grain fibre, and to a lesser extent fruit and vegetable fibre. However, insoluble grain fibre compared with soluble grain fibre showed an enhanced protective effect on CRC of approximately 70%. The reduced risk shown with insoluble grain fibre in this study is approximately 20% greater than the studies previously described in this Chapter, which have shown an approximate 50% reduction in risk when fibre as a whole was investigated.

More recently, resistant starch, a subset of fibre, has been more strongly linked with a decreased risk of CRC (Cassidy et al., 1994) (Kritchevsky, 1995). As inferred by its name, resistant starch is largely resistant to break down in the gastrointestinal tract, remaining mostly intact until it reaches the colon (Englyst et al., 1992). The properties of resistant starch will be described in more detail in section 1.4.2. In 2004, Muir et al (Muir et al., 2004) conducted a dietary intervention study on 20 volunteers with a family history of CRC and demonstrated that diets high in resistant starch improved faecal indexes of colonic health, including faeces output and transit time. These results indicate that different types of fibre have different effects on the colon and that resistant fibre is likely to provide greater benefits compared with other fibre types. Similar results have been observed by a number of investigators when different fibre types have been utilised in animal trials. These have been described in more detail in section 1.7. To better understand why the protective effects of fibre appear to depend on the type of fibre consumed, several studies have focussed on understanding the mechanism of fibre action.

1.5 Putative mechanisms of fibre action

1.5.1 Fibre in normal colonocytes

In normal colonic epithelium, BuA is the major energy source, (Chapman et al., 1994) providing over 60% of the colonocyte's energy requirements, (Cummings and Macfarlane, 1997) and has been shown to stimulate cell proliferation (Scheppach et al., 1995). Upon uptake in normal colonocytes, BuA undergoes rapid β-oxidation to produce energy (Wachtershauser and Stein, 2000) (Pouillart, 1998) as seen in Figure 1.4.

1.5.2 Physical properties of fibre reduce the risk of CRC

Initially, it was hypothesised that fibre rich diets gave a mild laxative type effect by reducing transit time and subsequently decreasing the exposure of the colonic epithelial cells to the carcinogens found in human faecal matter (Eastwood and Morris, 1992). This hypothesis was supported by the observation that increased faecal weight was associated with a decreased risk of CRC (Cummings and Stephen, 1980). The same author (Cummings and Stephen, 1980) also noted that dietary fibre had a number of other physical properties beneficial for preventing CRC including increased defecation frequency, shortened transit time, stimulation of beneficial microbial growth and dilution of colonic contents. However, in addition to the physical properties of fibre the study

also reported a number of other beneficial associations including the production of short chain fatty acids (SCFAs) by breakdown of fibre in the colon. Initially, SCFAs were thought to increase stool weight because they were poorly absorbed from the colon (Fordtran, 1971). However, it was later shown that SCFAs were in fact rapidly absorbed by the colon (McNeil et al., 1978) and that they have a short half life in the colon (Pouillart, 1998). The production of SCFAs will be discussed in more detail in section 1.6.1.

1.5.3 High fibre diets decrease colonic pH

In 1981, a hypothesis paper was published by Thornton (Thornton, 1981) which suggested that high fibre diets lead to an increased production of SCFAs and in turn a decrease colonic pH. In this paper, the author suggests that the geographical variation observed with CRC incidence is due to a difference in the pH of the colon and that this is caused by high and low fibre diets. The author describes that individuals eating high fibre low fat diets, for example Black Africans, had colonic pH values less than 6.5, compared with White Africans who had much higher colonic pH values and ate high fat low fibre Western diets. The pH values of each group also clearly corresponded to the respective incidence rates of CRC. Thornton then proposed that although the physical properties of fibre were clearly important, high SCFA production in the colon from high fibre intake would lead to decreased colonic pH and would as a consequence reduce the risk of developing CRC. More evidence was published to support this hypothesis by Topping and Clifton (Topping and Clifton, 2001) who demonstrated that SCFAs decreased colonic pH.

1.5.4 Fibre decreases secondary bile acid production

In addition to lowering colonic pH, a number of additional mechanisms of fibre action have been proposed. It has been demonstrated that some types of fibre can bind carcinogens (Smith-Barbaro et al., 1981) (Harris et al., 1993) and bile acids (Reddy et al., 1987) which may allow these compounds to pass out of the body in the faeces (Kim, 2000). Bile acids bound to fibre may prevent the conversion of primary bile acids to secondary bile acids (Kim, 2000), which are hypothesised to have a tumour inducing capacity (Reddy et al., 1987) (Venturi et al., 1997). Furthermore, the SCFA-induced decrease in colonic pH leads to a reduced solubility of free bile acids. This reduction in bile acid solubility is thought to decrease the tumour promoting activity of the secondary bile acids (Bruce, 1987). Additionally, an acidic pH of <6-6.5 has been demonstrated to inhibit the activity of the colonic bacterial enzyme 7 α-dehydroxylase, which is

responsible for the conversion of primary to secondary bile acids (Macdonald et al., 1978). Furthermore, acidification of faecal constituents has been hypothesised to prevent the constituent degradation of carcinogens (Harris and Ferguson, 1993).

1.5.5 Fibre modulates colonic bacterial populations

The human colon is estimated to have approximately 10^{14} bacteria with a metabolic activity similar to the liver (Hill, 1995). Dietary fibre can modulate colonic microflora by changing the predominant bacterial species, inducing functional changes or production of microbial enzymes important to carcinogen activation (Goldin and Gorbach, 1976). Additionally, dietary fibre has been shown to decrease the growth of anaerobic bacteria, which results in decreased production of secondary bile acids (Jacobs, 1988).

Fibre types which are broken down in the colon increase faecal bulk by increasing bacterial growth (Stephen and Cummings, 1980). The bacteria in the colon are the major water holding component of faeces (Stephen and Cummings, 1980), and high faecal water has been demonstrated to decrease the transit time of the stool. The combined increased faecal bulk and decreased transit time arising from increased bacterial growth reduce the exposure of the colonic mucosa to any carcinogens found in the faecal matter (Kim, 2000).

1.5.6 Summary of mechanisms of fibre action

Fibre has many effects on the colonic environment and all contribute to the reduced risk of CRC development. However, investigations into the SCFAs produced by high fibre diets have demonstrated that these molecules are likely to be one of the largest contributing factors to the decreased risk of CRC associated with high fibre intake.

1.6 SCFAs in the colon

1.6.1 Production of SCFAs

In addition to reducing colonic pH, it is now known that SCFAs are bioactive in the colon and they have also been demonstrated to play a major role in the maintenance of colonic homeostasis. As previously discussed, fibre is heterogeneous in nature with some components reaching the colon intact (Stephen et al., 1983) (Englyst and Cummings, 1985). Upon reaching the colon, the carbohydrates undergo breakdown by bacterial fermentation (Cummings et al., 1987). This fermentation is an anaerobic process which yields SCFAs including acetate, propionate and butyrate (BuA), various

gases including hydrogen, methane and carbon dioxide, and energy which is utilised by the bacteria (Cummings et al., 1987). Acetate, propionate and BuA are 2, 3 and 4 carbons in length respectively (Sengupta et al., 2006) and are produced in an average order of magnitude of acetate>propionate>BuA in the colon. The corresponding concentrations of SCFAs are thought to be approximately 60:25:10mmol/L with slight variations between different reports (Scheppach et al., 1995) (Miller and Wolin, 1979) (Grimble, 1989) (Rumessen, 1992). Both *in vitro* and *in vivo* studies have shown that the ratio of SCFA production is largely dependent on the type of fibre consumed (Scheppach et al., 1995) (Cummings et al., 1996) (Mortensen et al., 1991). As previously discussed, resistant starch, a type of fibre, has been linked more strongly with a decreased risk of CRC (Cassidy et al., 1994) (Kritchevsky, 1995) and has been shown to produce high concentrations of BuA in the colon (Topping and Clifton, 2001).

1.6.2 Physiological concentrations of SCFAs

Physiological concentrations of SCFAs in the colon vary considerably with the diet consumed, with studies reporting SCFA concentrations ranging from 60–150mM (Cummings et al., 1987) (Scheppach et al., 1988) (Hill, 1995). BuA represents approximately 15% of the SCFAs in the colon and reaches concentrations of up to 20mM in the colon and faeces of humans and animals (Newmark et al., 1994) (Wachtershauser and Stein, 2000) (Cummings et al., 1987) (Scheppach et al., 1988) (Hill, 1995) (Sengupta et al., 2006). In Westernised countries, SCFAs constitute approximately 5–15% of the total energy uptake and may be much higher in less developed countries (Wachtershauser and Stein, 2000). Evidence to support the role of SCFAs in the prevention of CRC has been demonstrated by a number of groups which show that high SCFA concentrations in faeces are linked to a decreased risk of CRC (Cummings et al., 1996). Furthermore, faecal BuA concentrations have been reported as lower in patients with colorectal cancer compared to healthy controls (Cassidy et al., 1994). Despite these findings, the mechanism by which BuA exerts its beneficial effects is still largely unknown.

1.6.3 SCFAs, in particular BuA, have anti-cancer properties

In the late 1970s to early 1980s, several groups demonstrated that SCFAs induced physiological effects on cells grown *in vitro.* In particular, BuA was shown to be a potent differentiation agent (Leder and Leder, 1975) (Leavitt et al., 1978) (Kruh, 1982b) (Whitehead et al., 1986). Since that time, BuA has been demonstrated to be the most bioactive when compared with acetate and propionate (Emenaker et al., 2001)

(Comalada et al., 2006) (Siavoshian et al., 1997). In CRC cell lines, BuA, at physiologically relevant concentrations, has been convincingly demonstrated to decrease proliferation, induce differentiation and increase apoptosis (Hague et al., 1993) (Heerdt et al., 1994) (Gamet et al., 1992) (Bartram et al., 1993) (Gibson et al., 1992) (Toscani et al., 1988). Experiments investigating the effects of BuA on *in vitro* CRC apoptosis and proliferation have been investigated in the current study in Chapter 3. Therefore, a detailed description of the BuA-induced changes in differentiation, proliferation and apoptosis will be discussed in that Chapter.

The evidence for BuA as an anti-CRC agent *in vitro* is vast and encompassing, as discussed in a number of experimental and review papers (Gamet et al., 1992) (Hague et al., 1993) (Heerdt et al., 1994) (Cummings et al., 1996) (Johnson, 1995; Johnson, 2002), however *in vivo* studies that have investigated the protective effects of resistant starch, BuA and its derivatives have yeilded mixed success.

1.7 *In vivo* evidence for the protective effects of resistant starch and BuA

As with all animal trials, it is imperative to have a model that is representative of the human disease or condition. Many of the animal studies utilised to examine the effect of diet on CRC have used the chemical carcinogens dimethylhydrazine (DMH) or azoxymethane (AOM). These chemicals induce the dysplasia-carcinoma sequence in rats (Hu et al., 2002) and lead to tumours of the colon and rectum.

McIntyre et al (McIntyre et al., 1993) investigated 3 types of dietary fibre with different fermentability to determine the effects of each fibre type on the luminal environment in DMH treated rats. They included measures of pH, SCFA concentrations, tumour size and incidence, and demonstrated that dietary fibres high in insoluble fibre produced greater concentrations of SCFAs in the colon and that this was related to a decreased tumour incidence and size.

Sakamoto et al (Sakamoto et al., 1996) investigated the effects of resistant starch and cellulose diets on DMH treated rats and found no change in carcinogenesis despite demonstrating an increased BuA concentration with high resistant starch diets. However, this group reported the use of high levels of DMH and suggested that this may have potentially caused the absence of any protective effect of resistant starch.

In a study by Le Leu et al (Le Leu et al., 2007) AOM-treated rats fed high resistant starch diets were shown to have a reduced incidence of colon adenocarcinomas. In another report by the same authors (Le Leu et al., 2003), the mechanism of resistant starch protection against CRC development was attributed to an increased apoptotic response in animals fed diets high in resistant starch. Similar results were observed by Bauer-Marinovic et al (Bauer-Marinovic et al., 2006) who demonstrated that hydrothermally treated (cooked) resistant starch prevented DMH-induced colon carcinogenesis by enhancing apoptosis of damaged cells. Apoptosis is an important regulator of colonic homeostasis, and the ability of resistant starch to induce this process in damaged cells through increased BuA concentrations in the colon is likely to be one of the underlying mechanisms responsible for the preventative effects of fibre on CRC. This concept was supported by Hu et al (Hu et al., 2002) who demonstrated that the apoptotic index of rats fed the poorly fermentable methylcellulose was much higher than those fed the more fermentable wheat bran following exposure to AOM. SCFA concentrations, including BuA, were higher in the rats fed MC compared with WB and it was hypothesised that BuA may play an important role in regulating apoptosis following genotoxic insult. Perrin et al (Perrin et al., 2001) investigated the factors required for the long-term stable production of BuA throughout the colon in relation to CRC risk. In rats they found that along with the type of fibre, the length of time that this fibre was consumed was a critical factor in the production of stable, high BuA concentrations.

In contrast, there are studies which show that increased SCFA concentrations do not protect against tumour development in rats exposed to CRC producing carcinogens (Sengupta et al., 2006). Zoran et al (Zoran et al., 1997) investigated the effect of wheat bran and oat bran on the size and incidence of CRC tumours in AOM treated rats. They demonstrated that rats which were fed wheat bran had significantly fewer tumours compared to the rats fed oat bran. This difference occurred despite oat bran producing significantly higher concentrations of BuA in the proximal and distal colon compared to wheat bran. Interestingly, in rats fed oat bran the ratio of acetate:propionate:BuA production was 45:15:35mmol/L, which is higher that the usually reported 60:25:10mmol/L. Experimental and methodological differences may explain the varied results (Perrin et al., 2001) (Sengupta et al., 2006) and, as previously described, the heterogeneous nature of fibre may also attribute to the conflicting findings. As discussed, fibre consumption changes many aspects of the luminal environment including pH, SCFA production, microbial growth, the predominant bacterial species, enzyme activity, transit time and thus exposure to carcinogens. Therefore, the different

results from individual studies have been attributed to the complexity of the effects induced by fibre intake (Sengupta et al., 2006). Consequently, investigation into the exact mechanism of BuA action is imperative to our understanding of the complex environment in the colon and the interactions of BuA in these conditions.

1.8 Mechanisms of BuA action

In CRC cell lines grown *in vitro*, BuA has been demonstrated to regulate proliferation, apoptosis, and differentiation and as a consequence much research has been conducted to understand the effects of BuA on each pathway. The biochemical mechanisms regulating both the proliferative and apoptotic pathways are extremely complex so prior to discussing the effects of BuA on each pathway it is important to describe the mechanism of apoptosis and proliferation. Simplified diagrams and descriptions have been presented in this Chapter to explain the key components of each pathway.

1.8.1 Proliferation

The cell cycle is controlled by 3 families of proteins; cyclins, cyclin-dependent kinases and inhibitors of cyclin-dependent kinases (Sherr, 1996). As shown in Figure 1.5, different cyclins are sequentially expressed during the cell cycle. Different cyclin-dependent kinases specific to each phase of the cell cycle are activated when bound to a particular cyclin (Blottiere et al., 2003) (Norbury and Nurse, 1992).

Inhibitors of cyclin-dependent kinases fall into 2 families: the Cip/Kip family and the INK4 family (Figure 1.5). The Cip/Kip family bind to and inhibit the activity of the complex formed between cyclins and cyclin-dependent kinases. Members of the Cip/Kip family known to be involved in cell cycle regulation include p21/Cip1, p27/Kip1 and p57/Kip2 (Blottiere et al., 2003) (Sherr, 1996).

The INK4 family of proteins modulate cell cycle by binding to cyclin-dependent kinases 4 and 6, which in turn prevents the binding of cyclin D (Figure 1.5). The complex formed between cyclin D and cyclin-dependent kinases 4 and 6 occurs early in the G1 phase and is crucial for the transition of the cell cycle as this complex is required to phosphorylate the retinoblastoma protein (Sherr, 1996). The phosphorylated retinoblastoma protein activates the E2F transcription factor and allows the transcription

of key genes required for progression out of the G1 phase of the cell cycle (Sherr, 1996) (Blottiere et al., 2003).

1.8.2 Apoptosis

It is evident by the high degree of conservation across different phyla that apoptosis in multicellular organisms is fundamentally important (Johnson, 2001). Figure 1.6 shows a simplified diagram of the molecular mechanisms of apoptosis. The two main pathways of apoptosis are induced either by cell surface receptors, indicated in this diagram as Fas, APO1 and CD95, or via the mitochondria. Apoptosis is mediated by "caspases", a family of cysteine proteases that exist in the cells as inactive zymogens. They require cleavage for their activation, which can often occur via the proteolytic activity of other caspases as a part of a signalling cascade. Caspase 8 is termed an initiator caspase as it reacts to extracellular signals, such as ligand binding to cell surface receptors, to initiate the caspase signalling pathway. Upon ligand binding to the Fas receptor, the formation of the Fas-associated death domain (FADD) is triggered, which in turn prompts the caspase signalling pathway by activating caspase 8. The signalling cascade eventually initiates the effector caspases 3, 6 and 7, which have both cytoplasmic and nuclear targets (Johnson, 2002).

The second main pathway of caspase activation is mediated via the mitochondria and is triggered by cellular stresses, such as DNA damage, cytotoxic stress and loss of extracellular growth factors (Johnson, 2001). The pro-apoptotic gene Bax facilitates release of cytochrome C, which binds to apoptotic protease-activating factor 1 (Apaf-1) and pro-caspase 9. The complex formed leads to the activation of caspase-9, which also activates the effector caspases 3, 6 and 7 (Johnson, 2002).

There are a number of other proteins which modulate and regulate the apoptotic pathway. As previously described in section 1.1.3, p53 is a tumour suppressor gene that is involved in the regulation of both proliferation and apoptosis, and mutations in the p53 gene are commonly observed in CRC (Ilyas et al., 1999). There are also a number of inhibitors of this pathway that regulate the apoptotic process. Key inhibitors of apoptosis include the IAP (inhibitors of apoptosis), and Bcl2 and Bcl-xL proteins which suppress the release of cytochrome C as illustrated in Figure 1.7.

1.8.3 BuA-induced changes to apoptosis and proliferation

To better understand the biological mechanisms of BuA action on proliferation and apoptosis, a number of groups have investigated the expression of cell cycle regulatory proteins and proteins modulating apoptosis following exposure to BuA. CRC cell lines exposed to BuA has been shown to induce changes in a vast number of genes and proteins as evident in publications which have used cDNA microarrays or protein analysis (Della Ragione et al., 2001) (Tabuchi et al., 2002). Mariadson et al (Mariadason et al., 2000a) investigated 8000 genes and demonstrated a change in 589 genes in SW620 CRC lymph node metastases after 48 hours exposure to 5mM BuA. Daly et al (Daly et al., 2006) investigated 19,400 genes in HT29 cells following 5mM BuA exposure for 24 hours and reported that nearly 2000 genes showed differential expression. Similar studies have been conducted using rat (Germann et al., 2003) and mouse samples (Tabuchi et al., 2006) again reporting large numbers of differentially expressed genes. This type of response to BuA is also observed at the protein level with Tan et al in 2002 and 2006 (Tan et al., 2006; Tan et al., 2002) using 2D gel electrophoresis to demonstrate differential expression of proteins from HT29 and HCT116 cells following 5mM BuA exposure. Although a large proportion of the differentially expressed genes and proteins have not been identified, it has been conclusively demonstrated that many are linked with apoptosis, proliferation, and cell cycle regulation and signalling.

1.8.4 Regulation of pro-apoptotic and anti-proliferative genes by BuA

Increased proliferation coupled with resistance to apoptosis is the hallmark of cancer cells (Hanahan and Weinberg, 2000) (Sherr, 1996). BuA-induced decreases in proliferation have been linked to cell cycle arrest in the G1 phase and the induction of terminal differentiation (Leder and Leder, 1975) (Siavoshian et al., 1997), with the addition of BuA to CRC cells leading to an increase in the relative number of cells in the G1 phase of the cell cycle (Siavoshian et al., 2000) (Blottiere et al., 2003). BuA-induced cell cycle arrest has been shown to occur via the up-regulation of genes involved in cell cycle regulation.

The role of both Cyclin D and p21/Cip1 has been outlined in Figure 1.6 and the importance of the p21/Cip1 protein in BuA-induced changes to proliferation was demonstrated by Archer et al (Archer et al., 1998b). Using the [^{3}H]Thymidine incorporation assay and HT29 cells transfected to overexpress the p21/Cip1 gene, they demonstrated that the over-expression of p21/Cip1 reduced cell proliferation.

Furthermore, this group used a HCT116 cell line in which p21/Cip1 had been deleted to demonstrate that the expression of this protein was required for BuA to induce a decrease in cell proliferation.

The expression of p21/Cip1 and cyclin D in response to BuA has been investigated by Siavoshian et al (Siavoshian et al., 2000). BuA was shown to inhibit the expression of both genes, although the mechanism by which BuA exerted these effects was unique to the gene investigated. BuA decreased the expression of cyclin D1 at the mRNA level without affecting its protein expression. Contrary to this, BuA stimulated the protein expression of cyclin D3 without affecting its mRNA expression. BuA was also shown to increase p21/Cip1 expression both at the mRNA and protein level.

As previously discussed in section 1.8.1 and in Figure 1.5, cells require retinoblastoma protein for progression out of the G1 phase of the cell cycle (Williams et al., 2003). Schwartz et al (Schwartz et al., 1998) reported that 2mM BuA treatment of LS174 CRC cell lines induced dephosphorylation of the retinoblastoma protein leading to cell cycle arrest. The same group also demonstrated that BuA treatment inhibited thymidine kinase activity, which is required for nucleic acid synthesis in the S phase of the cell cycle. A similar finding was reported in 1993 by Gope and Gope (Gope and Gope, 1993) who demonstrated a decrease in the phosphorylation of the retinoblastoma protein in the HT29 cell line following the addition of BuA. Expression of the tumour suppressor gene p53 is also modulated by BuA exposure, with Gope and Gope (Gope and Gope, 1993) demonstrating that the expression of p53 was down-regulated by approximately 6-7 fold following BuA treatment.

Increased expression of E-cadherin and alkaline phosphatase, both markers of differentiation, was observed in BuA-treated CRC cell lines (Butt et al., 1997). The pro-apoptotic marker BCL-2 homologous antagonist/killer (BAK) has been shown to be up-regulated with BuA treatment, with increased gene expression leading to greater caspase-3-mediated cleavage of target proteins, including poly-(ADP-ribose) polymerase (PARP) (Ruemmele et al., 1999) (Clarke et al., 2001), p21/Cip1 (Chai et al., 2000) and APC (Williams et al., 2003).

1.8.5 BuA modulates gene expression by interactions with the genome

From the studies described in section 1.8.3- 1.8.4 it is clear that BuA modulates the expression of many genes associated with the regulation of proliferation, apoptosis and

differentiation although the exact mechanisms remain unclear. Many of the effects of BuA action have been associated with alterations of nuclear chromatin structure (Smith et al., 1998) (Siavoshian et al., 1997). Chromatin is the structure responsible for the organisation and compacted nature of DNA in the nucleus, as well as playing a pivotal role in basal levels of gene expression. Chromatin is composed of histones, which are highly conserved, positively charged proteins that allow tight binding of the negatively charged DNA molecule (Ausio and van Holde, 1986). Alterations to this structure allow the DNA to bind less securely, enabling access to transcription factors and other regulatory enzymes and leading to increased specific gene transcription and expression (Svaren and Chalkley, 1990). Histone deacetylase (HDAC) is an enzyme which deacetylates the chromatin, thus keeping gene transcription repressed (Johnstone and Licht, 2003). BuA is an inhibitor of HDAC (Johnstone and Licht, 2003) (Chen et al., 2003), resulting in hyperacetylation and subsequent loosening of the histones (Candido et al., 1978; Vidali et al., 1978) allowing for increased accessibility to DNase 1 and other transcriptional factors (Svaren and Chalkley, 1990) and ultimately enhancing gene transcription.

Hyperphosphorylation of histones has also been implicated in the progression of cell cycle from one stage to another. Hyperphosphorylation of histone H1 has been demonstrated to promote chromatin condensation, which is a vital step in the progression of the cell cycle (Reeves, 1992). Along with its actions on histone acetylation status, BuA also acts to reduce the phosphorylation of H1, causing subsequent cell cycle arrest in the G_1-S phase (Reeves, 1992).

The major mechanisms that have so far been identified in BuA-induced effects have been presented in this Chapter, however there are also many other pathways which have been implicated in BuA-induced effects that have not been described in this report. For a more comprehensive review refer to Johnson et al (Johnson, 2002) and Scheppach et al (Scheppach et al., 1995).

1.9 BuA appears to have a paradoxical effect

As described, BuA has been shown to induce terminal differentiation, decrease proliferation and increase apoptosis of colon cancer cells. However, as described in section 1.5.1, BuA is the major energy source in normal colonic epithelium (Chapman et al., 1994) and has been shown to stimulate proliferation (Scheppach et al., 1995). Therefore, it is evident that BuA has two contrasting roles: the first role as an energy

source for normal colonic epithelium and the second as an inducer of differentiation and apoptosis in malignant cells (Hague et al., 1996).

1.9.1 Possible mechanisms underlying the apparent paradoxical effect of BuA

Despite advances in the understanding of BuA's molecular pathway an interesting question still remains; how does BuA seemingly promote 2 opposing pathways? That is, how does it act as an energy source and promote proliferation in normal cells yet in cancerous cells inhibit proliferation, induce differentiation and apoptosis? Jass (Jass, 1985) hypothesised that a shift from aerobic to anaerobic metabolism may occur during colorectal carcinogenesis as a result of a low fibre diet. Since the proposal by Jass in 1985, other authors have also proposed similar hypotheses whereby a switch from 'BuA-driven' to 'glucose-driven' metabolism occurs with cancer transformation (Boren et al., 2003) (Lambert et al., 2002). This hypothesis is supported by the increasing evidence that demonstrates dysregulation of energy homeostasis is associated with colorectal carcinogenesis (Gunter and Leitzmann, 2006).

In the absence of adequate fibre intake, low concentrations of BuA will be available as energy for colonocytes. The colonocytes would, therefore, be required to "switch" or adapt to utilise another energy source. It has been suggested that a shift in the type of metabolism in the colon would interfere with the oxidation of BuA (Jass, 1985) and malignant cells would become unable to metabolise BuA. If high concentrations of BuA are re-introduced the malignant cells may no longer be able to metabolise the BuA and this would result in an accumulation of BuA within the cell cytoplasm (Jass, 1985). This may reach toxic levels and could explain why malignant cells are sensitive to BuA when normal colonocytes are not.

1.9.2 A switch between energy sources may explain BuA's paradoxical effects

Malignancies have an accelerated metabolism whereby high glucose requirements and increased glucose uptake is observed (Macheda et al., 2005). The increased glucose uptake is thought to occur via an up-regulation of high affinity glucose transporters (Macheda et al., 2005) (Li et al., 2006), with Yamamoto et al (Yamamoto et al., 1990) demonstrating up-regulated Glut 1 expression in CRC. Baron-Delage et al (Baron-Delage et al., 1996) demonstrated an increase in Glut 1 expression in CaCo2 cells which had been transfected with the polyoma middle T (PyMT) oncogene . This leads to the suggestion that malignant cells would be less dependent on BuA as an energy source and, therefore, more reliant on glucose.

In HT29 cells, Boren et al 2003 observed that exposure to increasing concentrations of BuA lead to decreased glucose metabolism (Boren et al., 2003). This observation corresponded to a decrease in Glut 1 receptor expression detected by Li et al in a similar experiment (Li et al., 2006). Furthermore, Cuff et al demonstrated in HT29 cells that the BuA transporter, Monocarboxylate transporter 1 (MCT1), was up regulated with BuA exposure (Cuff et al., 2002). Lambert et al (Lambert et al., 2002) showed that MCT1 expression decreased with the transition to malignancy in colon tissue and observed a corresponding increase in Glut 1 receptor expression. This lead Lambert and colleagues to hypothesise that the cells had shifted from BuA to glucose metabolism. In 2003, Boren et al (Boren et al., 2003) provided more definitive evidence to demonstrate that HT29 cells are able to change from BuA to glucose-driven metabolism depending on the energy source available. This was achieved by measuring the uptake of radioactively labelled BuA and glucose in different cellular media environments.

1.9.3 What causes colonocytes to 'switch' from BuA to glucose-driven metabolism

Continual consumption of excess energy, particularly glucose, over prolonged periods of time causes the body to develop insulin resistance, leading to diabetes and hyperinsulinemia, hyperglycemia and may include glucose intolerance, hypertriglyceridemia and increased plasma non-esterified fatty acids (Bruce et al., 2000) (Gunter and Leitzmann, 2006). The continual high levels of energy and subsequent insulin resistance causes glucose to become available to organs and tissues, including the colon, which would not normally be exposed, and this situation is summarised in the diagram adapted from Bruce et al (Bruce et al., 2000) (Figure 1.7). Excess energy consumption and a low fibre diet are both typical of Western society diets, and together may be sufficient to induce a situation in the colon of high glucose and low BuA concentrations. As described by Jass (Jass, 1985), Boren et al (Boren et al., 2003) and Lambert et al (Lambert et al., 2002), in the absence of BuA the cells may be forced to switch to an anaerobic, glucose-driven metabolism. In the publication by Boren et al (Boren et al., 2003), the authors state that normal epithelium transition to malignant HT29 cells requires a prompt adaptation of the cells to a nutrient environment without BuA (Boren et al., 2003). Therefore it can be hypothesised that the potential of colon cells undergoing malignant changes may be determined by the type of energy available to those cells.

1.9.4 Nutrient uptake in the colon

As the availability of nutrients in the colon may be crucial to the malignant potential of the cells, it is vital to understand the energy regulating mechanisms, such as sensors and transporters. In an endeavour to better understand the mechanism of BuA action, many investigators have focused on investigating the transport mechanism for BuA into cells. Although the pH of the colon is dependent largely on diet, up to 95% of SCFAs are thought to exist in their ionized form (Cummings, 1987) (Tyagi et al., 2002). This renders the SCFAs unable to readily diffuse passively through the cell membrane, suggesting that the entry of BuA into cells must occur through an active uptake mechanism (Tyagi et al., 2002) such as transporters, channels or receptors coupled to these transport mechanisms.

A number of BuA transporters have been identified, including MCT1 (Ritzhaupt et al.) (Cuff et al., 2002) (Daly et al., 2005) (Cuff and Shirazi-Beechey, 2005) and more recently the SL5A8 gene, now described as the sodium-coupled monocarboxylate transporter (SMCT) (Rodriguez et al., 2002) (Li et al., 2003) (Coady et al., 2004) (Ganapathy et al., 2005). The hypothesis that BuA entry into the cell is via an active uptake mechanism is supported by Stein et al (Stein et al., 2000) who used ^{14}C- labelled BuA to show a decreased BuA uptake in the presence of the MCT1 inhibitors alpha-cyano-4-hydroxycinnamate and phloretin. This group also demonstrated that the uptake of BuA into CaCo2 CRC cells was enhanced by lowering pH. Cuff et al (Cuff et al., 2005) knocked down MCT1 expression in HT29 cells using RNAi, and showed that the ability of BuA to regulate the expression of key genes was abolished. They also showed that the knockdown of MCT1 was sufficient to alter BuA-induced changes to proliferation. Subsequently Daly et al (Daly et al., 2005) provided key information by measuring BuA-responsive genes in cell lines with low MCT1 expression. They identified a number of genes which were deregulated with cancer, and demonstrated a correlation between deregulation of key genes and lower expression of MCT1. Daly et al (Daly et al., 2005) then concluded that the transport of BuA into colonocytes underpins BuA's regulatory ability.

1.9.5 BuA sensors may regulate colonic homeostasis by allowing cell adaptation to nutrient availability

It is known that the concentration of BuA and other nutrients in the colon varies markedly depending on diet, with Mortensen et al 1991 (Mortensen et al., 1991)

demonstrating that the amount and ratio of SCFA's are highly dependent on the substrates consumed. Therefore, the ability of the cells in the colon to sense and adapt to fluctuations in available nutrients is imperative to normal colonic function. There is no question that even though BuA may affect many genes it is imperative to understand the mechanism of BuA entry into cells in order to comprehensively investigate the beneficial effects of BuA on CRC. It is also equally important to consider that BuA may not cross the membrane to exert some of its effects. Rather, BuA may interact with a "BuA sensor" on the surface of the cell membrane to then produce the observed effects. It is also possible that a BuA sensor may be linked to a BuA transporter or other protein which mediates uptake.

Dyer et al (Dyer et al., 2003a) demonstrated that glucose sensing, and thus the regulation of glucose uptake in the intestine, was via a glucose sensor on the external face of the luminal membrane linked to a glucose transporter. Glucose binds to the sensor and generates an intracellular signal leading to the up-regulation of the sodium/glucose (Na^+/glucose) co-transporter (SLG1). SLG1, located in the brush border membrane of the intestine, which is responsible for the transportation of D-glucose and D-galactose into the cells. In pre-ruminant lambs, milk sugar lactose is metabolised to D-glucose and D-galactose, which are both transported by SLG1. When the lambs are weaned, the diet consists largely of grass which does not produce high levels of D-glucose or D-galactose, and a 50-fold decrease in the levels of SLG1 are observed at both the mRNA and protein level. This decrease in expression is reversible with the administration of glucose, and is also seen in the intestinal cell line STC-1, where media containing high glucose concentrations induces SLG1 expression. Exposure of the STC-1 cell line to the 8-bromo-cAMP, a protein kinase A agonist, also induced an increase in SLG1 expression, as did exposure to the G-protein inhibitor pertussis toxin (PTX). Therefore, Dyer et al (Dyer et al., 2005) were able to show that the sensing of glucose did not rely on SLG1 itself, but rather a sensor. They then went on to show that this sensor was a G-protein coupled receptor (GPCR) by utilising inhibitory PKA/cAMP agents.

The metabolism of glucose is well defined with a large number of glucose transporters, sensors and other proteins shown to mediate the uptake and metabolism of glucose. However, an understanding of the receptor, sensor and transport system of BuA is still in its infancy. It is probable, that like the glucose regulatory system, the metabolism of BuA by colonocytes is equally complex.

1.10 G-protein coupled receptors may act as sensors to regulate the effects of BuA

In 1997, Velazquez et al (Velazquez and Rombeau, 1997) hypothesised that GPCRs were involved in mediating the effects of BuA on CRC cells. It was approximately 6 years later that the first 2 papers were published describing previously orphan GPCRs as receptors for BuA and other SCFAs (Le Poul et al., 2003) (Brown et al., 2003). The structure, function and role of GPCRs, including the putative BuA receptor are described in the upcoming sections.

1.10.1 Role of GPCRs

GPCRs are the largest family of cell surface receptors, and the genes encoding them account for greater than 1% of mammalian genomes (Marinissen and Gutkind, 2001). Activation of these receptors occurs with a wide range of ligands, including growth factors, vasoactive polypeptides, chemoattractants, neurotransmitters, hormones, phospholipids, photons and odorants, (Gutkind, 2000) and even proteases, such as thrombin, which activates its receptor by cleaving off a portion of the amino terminus. The broad range of ligands or GPCR activators together with the expression of these receptors in almost all tissue and cell types illustrates the significant role of GPCR's in physiological and pathophysiological processes (Stadel et al., 1997) (Horn et al., 2000) (Heasley, 2001) (Arvanitakis et al., 1997).

1.10.2 Structure of GPCRs

Structurally, GPCR's are made up of 7 stretches of 25-35 predominantly hydrophobic residues that form a 7 transmembrane (7TM) helical structure motif linked by 3 intracellular and 3 extracellular loops (Figure 1.8). The 7TM domain is coupled to an intracellular G-protein complex. The secondary structure of GPCRs varies enormously, and the presence of various G-protein complexes accounts for the high level of specificity observed with GPCR ligand binding. There are a number of different G-proteins and subunits, and often the 7TM is coupled to multiple G-proteins, allowing multiple responses from the same receptor depending on the type of ligand binding (Klabunde and Hessler, 2002).

1.10.3 Ligand activation of GPCRs

Activation of GPCRs consists of ligand binding and subsequent activation of the G-protein complex. The inactivated G-protein complex is found on the inner surface of the

plasma membrane and consists of an α, β and Υ subunit with GDP bound to the α component (Figure 1.8). Upon ligand binding, the receptor attaches to the G-protein complex causing a conformational change that releases GDP and allows GTP to bind. This leads to an activation of the α subunit, causing it to break away from the other subunits and move along the plasma membrane to an effector protein (Horn et al., 2000). Effector proteins can be either enzymes, known as secondary messengers, or ion channels. GTP is then converted back to GDP and Pi and the inactive G-protein is reconstituted.

Different ligands are able to induce different responses on the same GPCR depending on the pathway that is activated. The response resulting from GPCR activation depends on several factors, including the type of G-protein and its subunits and which secondary messenger system is activated, as described below.

1. Activation of the adenyl cyclase

Activation of the adenyl cyclase enzyme leads to the conversion of ATP to cAMP plus pyrophosphate (PP_i). Elevated levels of cAMP stimulate the activation of protein kinase A (PKA), which in turn phosphorylates other specific cell proteins, leading to a programmed cellular response (Marinissen and Gutkind, 2001).

2. Activation of phospholipase C

Activation of receptors bound to the G-protein G_q activates phospholipase C (PLC), which is found on the inner plasma membrane. Upon its activation, PIP_2 is cleaved to become IP_3 and DAG. IP_3 mobilises Ca^{++} release from the endoplasmic reticulum. An increase in the presence of Ca^{++} and DAG activates protein kinase C (PKC), which is normally located in the cytosol but upon binding of Ca^{++} its phospholipid binding site is exposed causing it to bind to the membrane. Upon activation PKC phosphorylates a variety of proteins, including some that are also activated by PKA (Marinissen and Gutkind, 2001). A description of different α-subunits and their downstream action along with compounds that can activate or inhibit each subunit can be found in Table 1.1.

Type of α-subunit	Action	Agonists and inhibitors
G_s	Stimulates adenylyl cyclase	Activated by cholera toxin
G_{olf}	Stimulates adenylyl cyclase	Activated by cholera toxin
G_{gus}	Stimulates adenylyl cyclase	Activated by cholera toxin
G_i	Inhibits adenylyl cyclase Activates Ca^{++} channels	Activation blocked by Pertussis Toxin
G_o	Activates K^+ channels Inactivates Ca^{++} channels	Activation blocked by Pertussis Toxin
G_q	Stimulates phospholipase C	Pertussis Toxin insensitive Activation blocked by GP2A
G_t	Stimulates cGMP phosphodiesterase in photoreceptors	

Table 1.1: Different α-subunits coupled to GPCRs, their actions when activated, and agonists or inhibitors that affect each subunit.

1.10.4 Abnormalities in GPCR expression and function have been linked to CRC

This superfamily of receptors is the primary mechanism for cells to sense environmental changes and, therefore, is essential in many biological processes (Gutkind, 2000). Some ligands to GPCR's are potent mitogenic agents and substrates that cause cell proliferation. Mutations in these receptors that lead to constitutive activation have been linked to cancer (Arvanitakis et al., 1997). Furthermore, locally produced ligands from cancers can act to further enhance or expedite tumourigenesis through GPCRs (Heasley, 2001). Abnormalities in G-protein expression and signalling along with over activation by ligand have been linked to a number of pathological diseases, including cancer (Horn et al., 2000). Continual over-activation of these receptors by ligand can often contribute to neoplasia, colon adenomas and carcinomas (Heasley, 2001).

1.11 GPCRs as putative BuA receptors

As the availability of nutrients and energy can fluctuate greatly, it is important to have mechanisms to constantly monitor or sense these changes. At the commencement of the current study in September 2003, SCFAs and fatty acids (FAs) were discovered to be the ligands for a previously orphan family of GPCRs; GPCR40, 41, 42 and 43 (Le Poul et al., 2003) (Brown et al., 2003) (Nilsson et al., 2003). This receptor group is closely related, and have a minimum of approximately 30% amino acid identity, and have few similarities to other GPCR families (Brown et al., 2003). The genes were isolated from a cluster on the chromosomal region 19q13.1 (Brown et al., 2003) (Le Poul et al., 2003). Despite their similarities, some of the GPCR40-43 receptors are coupled

to different α-subunits, and each SCFA binds with different affinities to each of the receptors.

1.11.1 GPCR40 does not bind SCFA

In the study by Briscoe et al (Briscoe et al., 2003) GPCR40 was shown to be activated by FAs with a carbon chain length greater than 6. This excluded BuA as a ligand, and consequently GPCR40 was not considered as a mediator for BuA-induced changes to apoptosis and proliferation.

1.11.2 GPCR42 is non-functional

Brown et al (Brown et al., 2003) demonstrated that GPCR42 was non-functional and hypothesised that due to the very high similarity to GPCR41, GPCR42 is a pseudogene of GPCR41 arising from a recent duplication event. Consequently GPCR42 was not investigated in the current study.

1.11.3 SCFAs bind to GPCR41 and 43

Using a ligand fishing strategy, Brown et al 2003 (Brown et al., 2003) showed that yeast with heterologous expression of human GPCR41 and 43 were activated by SCFAs in a specific and dose-dependent manner. Similarly, Le Poul et al (Le Poul et al., 2003) used the membranes of CHO-K1 mammalian cells, which had been transfected to express GPCR41 or GPCR43, and the [^{35}S]GTPγS assay to demonstrate SCFAs activated these receptors in a dose-dependent and specific manner.

In the report by Brown et al (Brown et al., 2003), GPCR41 bound SCFA with the affinities butyrate = propionate = pentoate > acetate > formate and in Le Poul et al (Le Poul et al., 2003) the affinities were propionate > isobutyrate > butyrate > valerate > isovalerate. For GPCR43, the SCFA affinities were reported by Brown et al (Brown et al., 2003) as butyrate = acetate = propionate > pentoate > hexoate = formate and by Le Poul et al (Le Poul et al., 2003) as propionate > butyrate = acetate > isobutyrate > caproate. Other 1-5 carbon length molecules with different structures were also tested using the same methodology with none being active against the receptors, indicating that it is the carboxylate anion which is active (Le Poul et al., 2003).

GPCR41 was discovered to be exclusively linked to the G-protein $G_{i/o}$ (Xiong et al 2004), whilst GPCR43 was shown to be linked to G_i, G_q and G_{12} α-subunits (Brown et al., 2003; Le Poul et al., 2003). The $G_{i/o}$ specific inhibitor, PTX, was utilised by both groups to

determine which of the α-subunits were coupled to GPCR41 and GPCR43. Brown et al (Brown et al., 2003) used a Calcium (Ca^{++}) mobilisation assay to measure intracellular Ca^{++} levels following SCFA exposure, whereas Le Poul et al (Le Poul et al., 2003) measured cAMP content of cells following treatment with SCFAs. In cells treated with either a vehicle or PTX, both groups demonstrated that the response of GPCR41 to SCFA activation was significantly blocked by PTX addition, whereas no change in response was observed with GPCR43. This result indicates that the GPCR43 signalling pathway is G_q-mediated not $G_{i/o}$-mediated as with GPCR41. However, in the report by Brown et al (Brown et al., 2003) it was demonstrated that ligand activation of GPCR43 in the [^{35}S]GTPγS binding assay using HEK293T cell membranes transfected to express GPCR43 can activate the $G_{i/o}$ family of proteins. This collectively suggests that GPCR43 can activate both the $G_{i/o}$ and G_q α-subunits.

As described, GPCR41 is most strongly activated by propionate> BuA >acetate, whereas GPCR43 is equally activated by each of the 3 SCFAs, with a slight preference for propionate (Karaki et al., 2006) (Brown et al., 2003) (Le Poul et al., 2003). Furthermore, of the 3 functional receptors in the GPCR40-43 family, BuA had the strongest effect on GPCR43. This makes it more likely that GPCR43 is the potential mediator of BuA-induced changes to CRC cells. BuA, compared to acetate or propionate, has been demonstrated to have a much greater effect on cancer cell proliferation, apoptosis and differentiation (Emenaker et al., 2001) (Comalada et al., 2006) (Siavoshian et al., 1997), and was, therefore, considered to be of most interest compared to the other SCFAs. Therefore, although an entire family of GPCRs were described as binding SCFAs and FA, only GPCR43 was investigated in this project for its involvement in mediating BuA-induced increases in apoptosis and decreases in proliferation.

1.11.4 Expression of GPCR43 in different tissue types

In the majority of published literature, GPCR43 expression was demonstrated using reverse-transcriptase PCR (RT-PCR) or quantitative-PCR (Q-PCR). The expression of GPCR43 is found most commonly in cells of the immune system, specifically leukocytes (Nilsson et al., 2003), peripheral blood mononuclear cells (PBMC), bone marrow (Brown et al., 2003) and spleen (Le Poul et al., 2003) (Nilsson et al., 2003). Recently, Karaki et al utilised immunohistochemistry techniques to demonstrate the expression of GPCR43 in rat (Karaki et al., 2006) and human (Karaki et al., 2007) colon.

1.11.5 GPCR43 as a putative BuA receptor, and mediator of apoptosis and proliferation

BuA has a number of physiological functions within the colon including the induction of apoptosis and decreased proliferation. Since this family of receptors bind FAs and SCFAs, it was hypothesised that these receptors may be mediators of BuA-induced changes to proliferation and apoptosis. The hypothesis was supported by other published data demonstrating that some effects of BuA are G-protein mediated. Davis et al (Davis et al., 2000) investigated the differentiation of the K562 erythroblast leukaemia cell line in the presence of BuA and found a significant increase in both the mRNA and protein expression of G_i, G_q, and G_β α-subunits. This group also established that the BuA-induced differentiation of this cell line could be blocked by the $G_{i/o}$ specific inhibitor PTX.

1.11.6 Function of GPCR43

Elucidating the function of GPR43 has been attempted in a limited number of publications, and although some interesting and exciting observations have been reported there is still no final conclusion regarding the exact biological role of GPCR43. Yonezawa et al (Yonezawa et al., 2006) used RNAi to demonstrate that functional GPCR43 was present in MCF7 breast cancer cell lines, although this work did not determine the function of this receptor. In a study by Senga et al (Senga et al., 2003), GPCR43 was shown to be upregulated with the differentiation of HL-60 and U937 leukemia cell lines into monocytes, which demonstrated a potential role for GPCR43 in cell differentiation. Two years later, Hong et al (Hong et al., 2005) conclusively demonstrated that GPCR43 was involved in the differentiation of 3T3-L1 adipocyte cells by knockdown of the receptor using RNAi.

The role of GPCR43 in SCFA modulation of gastrointestinal motility has also been examined by 2 groups. Peptide YY (PYY) and 5-hydroxytryptamine (5-HT) have been linked with SCFA-induced changes to colon motility. Karaki et al indicated a potential role for GPCR43 in SCFA-induced gastrointestinal motility by demonstrating co-localisation of GPCR43 with PYY-containing enteroendocrine cells and 5-HT-containing mucosal mast cells in rats (Karaki et al., 2006). In 2007, the same author also demonstrated co-localisation of GPCR43 with PYY-containing enteroendocrine cells in humans (Karaki et al., 2007). Investigating the possible role of GPCR43 in mediating colonic motility was conducted by Dass et al (Dass et al., 2007) using GPCR43 knock out mice (GPCR43-/-). This group demonstrated that SCFAs decreased the post

electrical field stimulation induced contraction of rat colon in organ baths. However, the response in wild type and GPCR43 null mice were not significantly different, suggesting that GPCR43 may not have a role in SCFA induced motility.

Hong et al (Hong et al., 2005) have also demonstrated that the expression of GPCR43 is altered with a change in the nutritional intake of rats. More specifically, rats which were fed high fat diets had a higher expression of GPCR43 in their adipose tissue compared to control rats fed a normal diet. The group described the "differential gene expression of GPCR43 in response to changes in nutritional status appeared to be an essential feature of the biological function of this receptor".

1.12 GPCR43 may act as a BuA sensor in the colon which mediates BuA-induced changes to apoptosis and proliferation

The regulation of energy metabolism is vital to cell survival (Dyer et al., 2005) and to maintain homeostasis, a balance between cell growth and cell death is required (Gavrieli et al., 1992). In the colon, the ability of epithelial cells to sense and consequently adapt to the constantly fluctuating dietary nutrient concentrations is essential for survival (Dyer et al., 2003b) and GPCRs have been implicated in nutrient sensing in the gastrointestinal tract (Dyer et al., 2003b). Hong et al (Hong et al., 2005) demonstrated that GPCR43 expression adapts to altered nutrient intake, which may indicate a possible role for GPCR43 as a nutrient sensor. Xiong et al (Xiong et al., 2004) used RNAi knockdown to show that activation of the GPCR41 with SCFAs induced the stimulation of leptin production from adipocytes. Leptin is an adipose derived hormone that regulates energy homeostasis (Xiong et al., 2004). Circulating leptin levels are tightly regulated and the work conducted by Xiong et al (Xiong et al., 2004) indicates GPCR41 modulates leptin production by sensing changes in SCFA concentrations. In 2003, Itoh et al (Itoh et al., 2003) demonstrated that GPCR40 was involved in sensing FA concentrations, and in the presence of high FAs, GPCR40 amplified glucose-induced insulin secretion in the pancreas. Itoh et al 2005 (Itoh and Hinuma, 2005) continued to investigate GPCR40 and concluded that GPCR40 may potentially be a cell-surface receptor for free fatty acids that regulates insulin secretion from pancreatic beta cells.

It was, therefore, hypothesised that GPCR43, like GPCR40 and GPCR41, may be a nutrient sensor and that GPCR43 was involved in sensing changes to SCFA

concentrations in the colon. As a result of this interaction with BuA, GPCR43 may also act to modulate BuA-induced changes to apoptosis and proliferation to ensure colonic homeostasis. This hypothesis is supported by recent reports published late in 2006 by Covington et al (Covington et al., 2006) and Karaki et al (Karaki et al., 2006) which suggest that the GPCR40-43 family may act as nutrient sensors.

1.13 Hypothesis

GPCR43 acts as a BuA sensor in normal and transformed colonic epithelium to enable colonocytes to sense and adapt to a changing extracellular environment (e.g. confluence and nutrient availability) to ensure colonic epithelium homeostasis by modulating BuA-induced changes to apoptosis and proliferation.

1.14 Aims

1. Determine if GPCR43 is present on a range of CRC cell lines derived from adenocarcinoma, carcinoma and metastatic disease.
2. Investigate whether the expression of GPCR43 is altered with various changes to the cellular environment.
3. Determine if GPCR43 expression is modulated by disease state using biopsies from patients' normal and malignant tissues.
4. Investigate whether GPCR43 is involved in mediating BuA-induced decreases in proliferation and increases in apoptosis.

1.15 Approach

To achieve the aims outlined in this project, the first series of experiments used consistent methodology to measure the proliferative and apoptotic response of 6 different CRC cell lines following exposure to BuA. Treatment with G-protein inhibitors was then conducted to determine if GPCRs were involved in the BuA-induced alterations in proliferation and apoptosis.

At the time of commencement of the experiments described, expression of GPCR43 had not been shown in colonic tissue. Therefore, confirmation of GPCR43 on CRC cells was achieved using reverse transcriptase PCR (RT-PCR). Once expression of the GPCR43 receptor had been confirmed, a quantitative PCR (Q-PCR) methodology was developed.

This Q-PCR technique was utilised to quantitatively measure changes in GPCR43 expression in a number of altered cellular environments, such as changes in nutrient availability, cell confluency and the development of resistance to BuA. When linked with measurements of apoptosis, proliferation and differentiation these experiments provided evidence to investigate the hypothesis that GPCR43 could function as a sensor for BuA.

Knockdown experiments, using RNAi techniques, were conducted to gain a greater understanding of the role of GPCR43 on BuA induced alterations in proliferation and apoptosis. Finally, a pilot study was conducted using human patient tissue samples to determine if GPCR43 expression changes in normal and malignant CRC disease states.

The experiments conducted in this thesis were designed to confirm the presence of GPCR43 in CRC samples and measure changes in receptor expression under various conditions to provide insights into the function of this receptor.

2 Materials and methods

Methods that were used repeatedly have been described in this Chapter. For more specific methodology refer to the relevant Chapters. For any experiments conducted with a kit, the relevant catalogue numbers have been referenced.

2.1 Maintenance of cell lines

Seven cell lines were used in this project; U937 monocytic cell line used as a control for RT-PCR experiments and 6 CRC cell lines chosen to represent the progression of CRC as shown in Figure 1.2. Of the cancer cell lines HT29 and SW480 cells are derived from an adenocarcinoma site, HCT116 and CaCo2 cells from carcinoma site and T84 cells from a lung metastasis. Each of the above CRC cell lines were obtained from sporadically occurring tumours, whereas LIM1215 cells, which are also from a carcinoma origin, are derived from a patient with HNPCC familial CRC. It is important to note that cell lines cannot be used to directly relate to stages of CRC in humans, however for the purposes of this thesis they have been categorised according to the ATCC (American Type Culture Collection) classification and have been ordered according to severity of disease. All tissue culture experimentation was conducted using aseptic techniques under sterile conditions and cell lines were maintained in a 37°C and 5% CO_2 Incusafe incubator.

Each of the cell lines were grown in their own unique media, and due to differences in growth rate, each required a different maintenance "split". A spilt is a subculturing of cells that are reaching confluence, whereby a small number of the cells from the confluent flask are diluted or "split" into a new flask to allow further growth without becoming over-confluent. As the CRC cell lines are adherent cells, when approximately 85% confluence was attained the cells were split by rinsing flasks in 10ml 1X PBS (Amresco, Aust.), adding 1ml of Trypsin/ETDA and returning the cells to the incubator for approximately 5 minutes to detach the cells. Once detached, the cells were suspended in appropriate media and the appropriate split; 1:5 for T84 and CaCo2, 1:10 for HT29, SW480, LIM1215 and 1:12 for HCT116 achieved by pipetting into a new T_{75} flask (Falcon, USA) containing 25ml of media. In contrast, U937 cells were grown in suspension; therefore detachment using Tyrpsin/EDTA was not required and an aliquot of cells in media was pipetted directly into a new T_{75} containing 25ml of media. A

maximum of 12 splits or passages were allowed before cells were disposed of and a new batch of cells processed from frozen stocks.

Cell stocks were stored in 1.5ml cyrovials (Nunc, Sweden) containing cells in Fetal Calf Serum (FCS) and 5% Dimethyl sulfoxide (DMSO) in liquid nitrogen (N_2) until required. Cyrovials containing cells were incubated briefly in a waterbath at 37°C to thaw the cells and were then pipetted into T_{75} tissue culture flasks (Falcon, USA) containing appropriate media. Cell lines were passaged to a maximum of 12 passages before being discarded.

Additionally, HT29 cells resistant to BuA (HT29-R) were produced by Dr Tanya Lewanowitsch by growing HT29 cells in the presence of increasing concentrations of BuA. Normal HT29 cells seeded into T_{75} flasks were initially exposed to filtered culture media (PALL 0.2uM syringe filter) containing 0.5mM BuA, with this media replaced twice per week. When the cultures reached approx 85% confluence, they were split 1:5 and allowed to grow media containing 0.5mM BuA for at least two splits. The BuA concentration was then increased by 0.5mM to 1mM and the culture again allowed to grow for a further two splits. This process was continued until a final 5mM BuA media concentration was achieved. Once the 5mM 'butyrate resistant' cell stocks were produced they were passaged to a maximum of 5 splits to maintain continuity in the cultures. A summary of the media components for each cell line appears in Table 2.1

Table 2.1: Description of CRC cell lines and growth media components.

Cell Line	Media
U937	RPMI 1640 medium (RPMI) (Gibco, Aust.) supplemented with 10% heat inactivated Foetal Calf Serum (FCS) (Gibco, Aust.), 1% Non Essential Amino Acids (NEAA) (Gibco, Aust.) and 1% Penicillin/Streptomycin (10,000 units of penicillin and 10,000 µg of streptomycin) (Pen/Strep) (Central Services Unit, Adelaide University)(CSU)
HT29 HT29-R	50% Dulbecco's Modified Eagle Medium (DMEM) (Gibco, Aust.) and 50% Ham's F-12 nutrient mixture (F-12) (Gibco, Aust.) supplemented with 5% FCS and 1% Pen/Strep. As above, except containing 5mM BuA (Sigma, USA)
SW480	RPMI medium supplemented with 10% FCS and 1% Pen/Strep
HCT116	McCoy's medium (Gibco, Aust.) supplemented with 10% FCS and 1% Pen/Strep
CaCo2	DMEM medium supplemented with 20% FCS, 1% NEAA 1% Sodium Pyruvate (Gibco, Aust.) and 1% Pen/Strep
LIM1215	RPMI medium supplemented with 10% FCS and 1% Pen/Strep
T84	50% DMEM and 50% F-12 nutrient mixture supplemented with 10% FCS and 1% Pen/Strep.

2.2 RNA extraction, purification and quantitation

2.2.1 Isolation of Peripheral Blood Mononuclear Cells (PBMC)

PBMC cells were used as a positive control in experiments as they have been previously shown to express high levels of GPCR43 (Brown et al., 2003). Nine ml of blood was obtained from a healthy human subject at the CSIRO Human Nutrition clinic. Ethics approval was obtained from the CSIRO human ethics committee. Blood was collected by venipuncture into sterile 10ml tubes. The blood was diluted 1:4 in sterile PBS and 36ml of the diluted blood placed into a 50ml tube (Falcon, USA) and carefully overlayed with 15ml of Ficoll Paque (GE Health Care Life Sciences, Aust.). The diluted blood was then centrifuged at 800g for 20 minutes, at which time the PBMC cells were localised at the interphase of the Ficoll Paque. The PBMC cells were then removed using a glass pasteur pipette (Chase Scientific Glass, USA) and the cells washed twice in 15ml PBS by centrifugation at 300g for 10 minutes. The cell pellet containing the PBMCs was then immediately processed for RNA isolation, as discussed in 2.2.2.

2.2.2 Isolation of Total RNA from in vitro cell lines and PBMC cells

For the adherent cell lines, total RNA was extracted from T_{75} flasks when the cells were approximately 85% confluenct. Cells were treated with trypsin and cell pellets collected by centrifugation at 300g for 10 minutes. For U937 cells, media containing cells in suspension was removed from T_{75} flasks and the cells pelleted by centrifugation at 300g for 10 minutes. All cell pellets, including PBMC cell pellets were processed for RNA isolation using the RNeasy mini kit (Qiagen, Aust. #74104) according to the manufacturer's instructions. Cells were lysed using in 350μl of RLT lysis buffer and the samples homogenised by loading the cell lysate into a QIAshredders column and centrifuged at 12,000rpm for 1 minute. An equal volume of 70% Ethanol (Merck, Aust) was added to the homogenised sample, the total sample added to an RNeasy spin column, and the sample centrifuged at 14,000rpm for 20 seconds to allow the RNA to bind to the silica membrane inside the column. The column was then washed twice with 350μl of RW1 buffer before an on column DNA digest was conducted. Briefly, 10μl of RNase free DNase was added to 70μl of RDD buffer, the total 80μl of this solution added directly to the silica membrane, and incubated at room temperature for 15 minutes. The spin column was transferred to a new collection tube, 500μl of RPE buffer added to the spin column and centrifuged at 14,000rpm for 20 seconds. A second RPE wash was conducted and the samples centrifuged at 14,000rpm for a 2 minute period to dry the membrane. The column was then transferred to 1.5ml tube, 50μl of nuclease free water added directly to the silica membrane and the column inside the 1.5ml tube centrifuged at 14,000rpm for 1 minute to elute the RNA. The RNA was then immediately processed to remove any genomic DNA (gDNA) which may have remained in the sample.

2.2.3 Additional gDNA digest of RNA from cell lines

Due to the intronless nature of GPCR43, it was necessary to remove any contaminating gDNA that may remain in the RNA samples that escaped the on column DNA digest described in 2.2.2. Therefore, an additional DNA digest was carried out according to manufacturer's instructions for DNA digestion found in the RNeasy Mini kit. Once the additional gDNA digest of RNA samples was completed, the RNA was stored at -80°C until required.

2.2.4 Quantification of RNA samples

For all RNA samples, 2μl of RNA was placed onto the NanoDrop-1000^{TM} (NanoDrop) (NanoDrop Technologies) and absorbance was recorded at 260nm and 280nm. Concentration was calculated by the software program, using the formula:

$O.D_{260}$ x dilution factor x 0.04 = RNA concentration in μg/μl

In addition the quality of the RNA was tested by calculating $O.D_{260}/O.D_{280}$ with values ≥ 1.8 accepted.

2.2.5 Electrophoresis of RNA samples

Gel electrophoresis tanks (Thermo EC classic tanks) were washed in RNAse Zap (Sigma, USA) to ensure no contaminating RNAses were present. 2μl of the RNA samples to be used in RT-PCR experiments were diluted in 8μl of 5x RNA loading buffer (50% glycerol (CSU), 0.1%TAE (CSU, Ad Uni), 1% w/v Bromophenol Blue (Sigma, USA), 1% Xylene Cyanol (Sigma, USA) in Diethyl pyrocarbonate MQ water which had been autoclaved (DEPC-MQ H_2O). The diluted samples were loaded into the wells of a 1% (w/v) agarose (Sigma, USA) gel in 1X TAE running buffer, then run at 100V for approximately 30-40 minutes. The New England Bioscience 2-log ladder was used as a size indicator. Gels were then stained in 1μg/ml Ethidium Bromide (Sigma, USA) for 10 minutes and destained in DEPC-MQ H_2O for 3 minutes. Gels were then photographed using a Kodak DC265 digital camera under U.V. light to visualise the RNA bands.

2.3 cDNA synthesis and RT-PCR

2.3.1 cDNA synthesis

cDNA was synthesised using the Invitrogen's Superscript II kit (#18064-022). All products described in this section were purchased from Invitrogen, Australia. 1μg of RNA was added to 1μl of oligo dT (500ug/ml) and 1μl of dNTPs (10mM) and made to a total volume of 12μl with molecular grade water (Invitrogen, Aust.). The solution was heated at 65°C for 5 minutes and then cooled on ice. 4μl of 5X first strand buffer, 2μl 0.1M DTT and 1μl (40 units) of RNase out were added and heated at 42°C for 2 minutes. Finally 1μl (200 units) of Superscript II was added and solution was mixed by pipetting upwards and downwards, then incubated at 42°C for 50 minutes. The enzyme

was inactivated by incubation at 70°C for 15 minutes. cDNA was diluted 1:10 with molecular grade water before addition to PCR reactions.

2.3.2 Oligonucleotide Primers used in RT-PCR

All primers were purchased from Geneworks (Aust.) and diluted to 1μg/μl in molecular grade water (Invitrogen, Aust.). The primer sequences used to amplify GPCR43 are as follows:

43FWD1	Sequence: 5'-CTGTGGTGACGCTGCTCAAT-3'
43FWD2	Sequence: 5'-ATGCTGCCGGACTGGAAGAG-3'
43FWD3	Sequence: 5'-ACGTGGTGCTGCCCGTGCGG-3'
43REV1	Sequence: 5'-CCAGACTGGCGTTGAGTGAA-3'
43REV2	Sequence: 5'-CTACTCTGTAGTGAAGTCCGAAC-3'

Each oligonucleotide pair was used to amplify specific regions of the cDNA generated. Each pair resulted in an amplicon of specific length. The following shows the placement of each primer along the nucleotide sequence of the GPCR43 gene.

1 atgctgccgg actggaagag ctccttgatc ctcatggctt acatcatcat cttcctcact
61 ggcctccctg ccaacctcct ggccctgcgg gcctttgtgg ggcggatccg ccagccccag
121 cctgcacctg tgcacatcct cctgctgagc ctgacgctgg ccgacctcct cctgctgctg
181 ctgctgccct tcaagatcat cgaggctgcg tcgaacttcc gctggtacct gcccaaggtc
241 gtctgcgccc tcacgagttt tggcttctac agcagcatct actgcagcac gtggctcctg
301 gcgggcatca gcatcgagcg ctacctggga gtggctttcc ccgtgcagta caagctctcc
361 cgccggcctc tgtatggagt gattgcagct ctggtggcct gggttatgtc ctttggtcac
421 tgcaccatcg tgatcatcgt tcaatacttg aacacgactg agcaggtcag aagtggcaat
481 gaaattacct gctacgagaa cttcaccgat aaccagttgg acgtggtgct gcccgtgcgg
541 ctggagctgt gcctggtgct cttcttcatc cccatggcag tcaccatctt ctgctactgg
601 cgttttgtgt ggatcatgct ctcccagccc cttgtggggg cccagaggcg gcgccgagcc
661 gtggggctgg ctgtggtgac gctgctcaat ttcctggtgt gcttcggacc ttacaacgtg
721 tcccacctgg tggggtatca ccagagaaaa agcccctggt ggcggtcaat agccgtggtg
781 ttcagttcac tcaacgccag tctggacccc ctgctcttct atttctcttc ttcagtggtg
841 cgcagggcat ttgggagagg gctgcaggtg ctgcggaatc agggctcctc cctgttggga
901 cgcagaggca aagacacagc agaggggaca aatgaggaca ggggtgtggg tcaaggagaa
961 gggatgccaa gttcggactt cactacagag tag

The primer sequences used to amplify the housekeeping gene β-actin and the MCT1 gene are as follows:

B-actin FWD	Sequence: 5'-CTGGCACCACACCTTCTA-3'
B-actin REV	Sequence: 5'-GGGCACAGTGTGGGTGAC-3'
MCT1 FWD	Sequence: 5'-GGCTGGGCAGTGGTAATTGGAGCT-3'
MCT1 REV	Sequence: 5'-GGCCCGATTGGTCGCATGAGGGCT-3'

2.3.3 RT-PCR

Each 25μl PCR reaction consisted of: 0.5μl of dNTP (10mM), 2.5μl NH_4 buffer (Bioline, Aust.), 1.5mM $MgCl_2$ (Bioline, Aust.), 1μl of forward primer (0.1μg/μl), 1μl of reverse primer (0.1μg/μl), 0.2μl (1 unit) of Taq polymerase (Bioline, Aust.) with total volume made up to 24μl using molecular grade water. A master mix of all reagents listed above was generated immediately prior to the PCR reaction to reduce errors associated with pippetting small volumes. 24μl of the master mix was added to 1μl of cDNA.

Cycling parameters were optimised for each primer set using a Hybaid thermocycler.

GPCR43 protocol consisted of:

94°C for 3 minutes

94°C for 30 seconds
55°C for 30 seconds
72°C for 60 seconds
} 40 cycles

72°C for 5 minutes
10°C for 10 minutes

β-Actin protocol consisted of:

94°C for 3 minutes

94°C for 30 seconds
60°C for 30 seconds
72°C for 30 seconds
} 40 cycles

72°C for 5 minutes
10°C for 10 minutes

2.3.4 Electrophoresis of RT-PCR products

5μl of PCR reaction solution was added to 1μl of DNA loading buffer (50% glycerol, 0.1%TAE, 1% w/v Bromophenol Blue, 1% Xylene Cyanol in MQ H_2O). The diluted samples were loaded into the wells of an agarose gel (1.5% (w/v) in 1X TAE) then electrophoresed at 100V for approximately 30-40 minutes. A New England Bioscience 100bp ladder was used as a size indicator. Gels were then stained in 1μg/ml Ethidium Bromide for 10 minutes and destained in DEPC-MQ H_2O for 3 minutes. Gels were then photographed using a Kodak DC265 digital camera under U.V. light to visualise the cDNA bands.

2.3.5 Densitometry analysis of gel electrophoresis

Densitometry analysis of gel electrophoresis photographs taken using the methodology described in 2.3.4 was conducted using Scion Image software.

2.3.6 Isolation of gDNA

Since GPCR43 is an intronless gene, gDNA can be used in PCR reactions to produce full length amplicons. These amplicons were used to create a standard curve for Q-PCR analysis, which will be described in 5.3.1. Furthermore, gDNA was also used in PCR reactions to ensure each cell line possessed a copy of the gene encoding GPCR43.

gDNA was extracted from T_{75} flasks of approximately 85% confluence using a DNeasy mini kit (Qiagen, Aust. #69504) according to manufacturers instructions.

2.3.7 Purification of gDNA used in RT-PCR

Following the PCR reactions and using gDNA as a template from 2.3.6, the QIAquick PCR purification kit (Qiagen, Aust. #28104) was used according to the manufacturer's instructions to remove any contaminating enzymes, salts or dNTP's left from the PCR reaction to enable subsequent PCR.

2.3.8 Quantitation of DNA

The concentration of the DNA produced from purified PCR reactions described above in 2.3.7 was measured by adding 2μl of DNA was added to the NanoDrop® ND-1000 UV-Vis Spectrophotometer (NanoDrop) and the absorbance was recorded at 260nm and 280nm. Concentration was calculated using the formula:

$O.D_{260}$ x dilution factor x 0.05 = DNA concentration in μg/μl

Once the concentration had been calculated, the samples were diluted and used to produce a standard curve for Q-PCR analysis. The methods are described in detail in 5.3.1.

2.4 Quantitation of GPCR43 using Q-PCR

2.4.1 Real-Time PCR

Commercially available pre-validated primer and probe sets were used to amplify GPCR43 (Applied Biosystems, USA #Hs00271142_s1) and the housekeeping genes 18S (Applied Biosystems, USA #Hs99999901_s1) and GAPDH (Applied Biosystems, USA #Hs99999905_m1)

Master mix solutions consisting of 12.50μl of Taqman Universal PCR master mix (Applied Biosystems, USA), 1.25μl of 20X assay on demand which contains the primers and fluorescent probe (Applied Biosystems, USA) and 11.25μl of molecular grade water were generated. 24μl of the master mix solution was added to 1μl of cDNA or 1μl of each dilution of the standard curve (the methods used for establishing the standard curve are described in 5.3.2) to give total reaction volumes of 25μl. Each reaction was conducted in triplicate in a total of 3 independent experiments. Reactions were amplified in a ABI 7300 Real-Time PCR System (Applied Biosystems, USA) with the following cycling parameters:

95°C for 10 minutes

95°C for 30 seconds
60°C for 1 minute
} 45 cycles for GPCR43 and 40 cycles for each housekeeping gene

72°C for 10 minutes

2.5 Determining changes in GPCR43 expression during changes in cell confluence, BuA exposure and BuA exposure with alternate energy sources

2.5.1 Photomicroscopy

Microscopic photos were taken at 5x and 10x magnification, to examine differences in cell morphology. Cells in T_{75} flasks were placed onto the stage of a Zeiss Axio vert 40LFL microscope and visualised using a Motic Cam 2000 2.0 mega pixel camera and Motic Images plus 2.0 software. Once the images were captured the cells were processed for RNA and subsequent Q-PCR reactions as described in sections 2.2.2-2.3.1 and 5.3.6.

2.5.2 Methods for determining changes in GPCR43 expression during changes in cell confluence, BuA exposure and BuA exposure with alternate energy sources

The expression of GPCR43 was investigated with a number of treatments and conditions including cell confluence and with or without the presence of BuA in high and low glucose media. The detailed methods are described in the relevant Chapters (6 and 7).

2.6 Measuring CRC cell proliferation/apoptotic responses

2.6.1 Proliferation and Apoptosis Assays: Cell Titre Blue™ Cell Viability Assay and Apo-One™ Homogenous Caspase 3/7 Assay

For each of the cell lines examined, flasks of 85% confluence were trypsinised, plated at 50,000 cells/well in black Fluro Nunc plates (Nunc, Sweden) and incubated at 5% CO_2, 37°C for 24 hours. After 24 hours the media was aspirated and cells were washed in 100ul of 1X PBS. 100µl of cells were treated with 1mM, 2.5mM, 5mM, 7.5mM, 10mM, 15mM and 20mM BuA in their respective media containing 3% FCS standardised for all cell types. Plates were then incubated at 5% CO_2, 37°C for a further 46 hours. At 46 hours, 20µl of Cell Titre Blue reagent (Promega, Aust. #TB317) was added, and plates incubated for a further 2 hours at 5% CO_2, 37°C. At 48 hours, plates were read on a Wallac Victor 3 plate reader (Perkin Elmer, Aust.) at excitation/emission of 550/615nm to obtain proliferation measurements. 100µl of Apo-One reagent (Promega, Aust. #TB295) was then added to each of the wells and the plates placed on a plate shaker at room temperature in the dark for a further 2 hours. The plates were then read on the plate

reader at excitation/emission of 485/535nm to obtain apoptosis measurements. 3 independent assays were conducted in triplicate and results pooled.

These assays were conducted with modifications and are discussed in the relevant Chapters.

2.7 RNAi Knockdown of GPCR43

To investigate the potential role of GPCR43 in BuA-induced changes to CRC cell proliferation, apoptosis and differentiation, RNAi knockdown of the receptor was conducted. The expression of GPCR43 was measured in cells treated with GPCR43 siRNA and all relative controls according to methods described in Chapter 8. Cells which had been confirmed to express lower levels of GPCR43 were used in apoptosis, proliferation and differentiation assays. The specific methods are described in Chapter 8.

3 BuA-induced proliferative and apoptotic response of CRC cell lines and the possible role of G-protein coupled receptors

3.1 Introduction

In *in vitro* studies, using CRC cells grown in culture, BuA has been demonstrated convincingly, at physiologically relevant concentrations, to decrease proliferation and induce differentiation, whilst increasing apoptosis in CRC cell lines (Hague et al., 1993) (Heerdt et al., 1994) (Bartram et al., 1993) (Gamet et al., 1992) (Gibson et al., 1992) (Toscani et al., 1988). However, in normal colonocytes, BuA is the preferential energy source (Roediger, 1980) (Csordas, 1996) and is rapidly metabolised (Miller et al., 1987) (Gaschott et al., 2001).

Many investigators have studied BuA-induced decreases in proliferation and increases in apoptosis, and therefore, a variety of different experimental techniques have been utilised to measure these effects. These include FACS analysis (Buda et al., 2003) (Marchetti et al., 1997), DNA fragmentation (Levy et al., 2003) (Haza et al., 2000) (Marchetti et al., 1997), measurement of caspase activity (Levy et al., 2003) (Hofmanova et al., 2005), ^{3}H-Thymidine incorporation (Archer et al., 1998a) (Emenaker and Basson, 2001), and counting floating cells with (Singh et al., 1997) and without (Bonnotte et al., 1998) specific apoptotic stains to determine if the floating cells are apoptotic or necrotic. Although many cell lines have been used throughout experimentation as indicators of CRC, each cell line is derived from an individual patient (or from the same patient at a different disease state) and consequently each will be from a different stage of CRC and have a distinct genetic makeup and morphology. This makes it likely that each cell line will behave in a unique manner. Therefore, whilst many research groups have investigated the effects of BuA on CRC cell lines, differences in the approach taken, cell line used and time of exposure to BuA has lead to variation in the results obtained from different studies and has complicated comparisons between these studies. For this study, 6 CRC cell lines were chosen to represent the adenocarcinoma, carcinoma and metastatic stages of CRC (Figure 1.2). The morphology, growth rates (as indicated by split), growth media and origin of each of these cell lines has been summarised in Table 3.1.

A comprehensive investigation of the effects of BuA on multiple, staged, CRC cell lines has previously been conducted by Hague et al in 1993 (Hague et al., 1993) and 1995 (Hague et al., 1995) who investigated the BuA-induced apoptotic response of 6 adenoma and 7 carcinoma CRC cell lines in 1993 (Hague et al., 1993) and 3 adenoma and 4 carcinoma CRC cell lines in 1995 (Hague and Paraskeva, 1995). Although this group measured apoptosis, they did not measure proliferation. The current study will build on the research conducted by Hague et al (Hague et al., 1993) (Hague and Paraskeva, 1995) as the Cell Titre Blue™ Cell Viability Assay and Apo-One™ Homogenous Caspase 3/7 Assay enables the measurement of both proliferation and apoptosis in the same cell population The value of CRC cell lines have been shown in experiments investigating the effects of BuA on cellular events. Additional value can be obtained by comparing the effects of BuA across different cell lines that reflect different stages of disease progression. The first major focus in this Chapter is that comparison.

Since discovering the anti-proliferative and pro-apoptotic effects of BuA on CRC cells, many studies have attempted to elucidate the mechanisms of BuA action. Although significant discoveries have been made, the exact pathway by which BuA induces these effects remains largely elusive. As described in Chapter 1, many reports have focused on investigating the transport of BuA into cells in an endeavour to better understand the mechanism of BuA action. The GPCR40-43 family provide potential candidates for involvement in the uptake or regulatory ability of BuA on CRC cells. This family of receptors were recently shown to be activated by SCFAs and FAs by 3 research groups (Brown et al., 2003) (Le Poul et al., 2003) (Nilsson et al., 2003). The current study aims to determine if this family of receptors, in particular GPCR43, plays a role in BuA-induced changes to apoptosis and proliferation. BuA has previously been associated with many different pathways and biochemical mechanisms in the cell, and therefore, the second major aim of this Chapter was to ascertain whether BuA-induced decreases in proliferation and increases in apoptosis were influenced by G-proteins. Blockade of G-protein signalling, using G-protein inhibitors, PTX and GP2A, was used in this study to identify any potential role of GPCRs in BuA-induced changes to apoptosis and proliferation.

PTX inhibits the $G\alpha_{i/o}$ subunit by blocking the ADP-ribosylation of the α-subunit, causing the α,β,Υ complex to remain bound to GDP, thereby stopping receptor activation following ligand binding (Wang et al., 2007). GP2A is a peptide that selectively inhibits the activation of $G\alpha_q$ subunit, again preventing receptor activation upon ligand binding

(McKillop et al., 1999). Both PTX and GP2A were used in this study as the GPCR40-43 receptor family has been shown to activate both the $G\alpha_q$ and $G\alpha_{i/o}$ subunits as previously described in Chapter 1.

3.2 Approach

1. To compare the proliferative and apoptotic response of 6 CRC cell lines following exposure to BuA.
2. To determine if these BuA effects are influenced by G-protein inhibitors in 2 cell lines, derived from an adenocarcinoma site (HT29) and a carcinoma site (HCT116).

3.3 Methods

3.3.1 Measurement of proliferation and apoptosis of 6 CRC cell lines

Refer to section 2.6.1 for methodology.

3.3.2 Proliferation and apoptosis in the presence of G-protein inhibition

HT29 cells and HCT116 cells were trypsinised, plated and incubated according to methods described in section 2.6.1. Following the 24 hour incubation after plating, media was aspirated and G-protein inhibitors in fresh media were added to yield final inhibitor concentrations in the range of 500ng/ml-2000ng/ml PTX and 5-10μM GP2A. The concentration ranges of PTX and GP2A were chosen as they were similar to those used successfully in a number of published studies (Carracedo et al., 1998) (Davis et al., 2000) (Hunt et al., 1999) (Choisy et al., 2004; McKillop et al., 1999; Tanski et al., 2004). Plates were then incubated in 5% CO_2 at 37°C for 1 hour. Following the 1 hour incubation, BuA was added to the G-protein inhibitor treatment to final concentrations in the range of 1mM-20mM BuA. Plates were then incubated in 5% CO_2 at 37°C and all protocols followed as detailed in methods described in 2.6.1 to determine the proliferative and apoptotic response.

3.3.3 Statistical analysis

For the response of each cell line to BuA exposure, One-Way ANOVAs with Tukey's post hoc testing were performed comparing all values within each cell line individually. For comparisons across cell lines, One-Way ANOVAs were performed with Tukey's post hoc testing comparing all values from all cell lines. Significance is indicated by values of

$p<0.05$. All statistical analyses were conducted using GraphPad Prism 5 software for Windows.

3.4 Results

3.4.1 Proliferation in 6 CRC cell lines in response to BuA exposure

Proliferation results are shown for all cell lines in Figure 3.1. Differences between the values recorded in the absence of BuA (0mM) for each of the cell lines demonstrate the variation in basal growth rates across these cell lines. LIM1215, HT29, SW480 and HCT116 cells showed a high basal growth rates, while the CaCo2 and T84 cells had the lowest growth rates over the 48 hour period as determined by a One-Way ANOVA with Tukey's Post Hoc testing.

HT29 cells displayed a gradual, dose dependent decrease in proliferation, which continued to fall with increasing concentrations of BuA. SW480 cell proliferation also decreased in a dose dependent fashion until exposed to concentrations of 7.5mM BuA or higher, when no further decreases in proliferation occurred. The magnitude of the decrease in T84 cell proliferation is much lower than the other cell lines, and was only significant at 7.5mM and 20mM BuA. In the HCT116 and CaCo2 cell lines, 2.5mM was sufficient to induce a maximal decrease in cell proliferation. LIM1215 cell proliferation was unchanged at 2.5mM BuA and then decreased in a dose dependent manner. Concentrations of 5mM or greater were sufficient to induce a maximal decrease in proliferation in the LIM1215 cells, in the same manner observed in the HCT116 and CaCo2 cell lines.

Results from this experiment have been summarised in Table 3.2, which shows the percentage decrease in proliferation relative to no BuA treatment for each cell line. For HT29, SW480 and T84 cells, the percentage decrease from maximum proliferation continued to decline with increasing BuA concentrations from 5mM to 20mM BuA. For HT29 and SW480 cells, the decrease from 5mM to 20mM was significant and although the values for T84 at 5mM and 20mM were not significantly different the same trend is still observed. In the HCT116, CaCo2 and LIM1215 cells the values at both 5mM and 20mM are very similar, indicating that maximal decreases in proliferation have occurred following 5mM BuA exposure.

% decrease in proliferation relative to basal proliferation (0mM BuA control)		
Cell line	**5mM**	**20mM**
HT29	35.6 ± 2.5	56.1 ± 3.3***
SW480	22.5 ± 1.4	37.1 ± 1.1***
HCT116	46.7 ± 6.9	47.4 ± 8.0
CaCo2	36.7 ± 0.9	41.1 ± 1.5
LIM1215	17.9 ± 4.7	25.9 ± 3.1
T84	17.3 ± 4.5	32.0 ± 3.6

Table 3.2: Percentage decrease in proliferation relative to 0mM BuA in each cell line at 5mM and 20mM BuA. (*) $p<0.05$ (**) $p<0.01$ (***) $p<0.001$ denotes a significant difference compared to 5mM BuA for the same cell line (One-Way ANOVA with Tukey's Post Hoc testing using values from each cell line respectively).

3.4.2 Apoptosis in 6 CRC cell lines following exposure to BuA

The apoptosis results obtained in this experiment are displayed in Figure 3.2. Differences between the cells in the values measured at 0mM BuA for each of the cell lines again demonstrates the variation in basal apoptosis rates across the different cell lines. SW480 cells have the highest basal apoptosis rates followed by the LIM1215 cells. The basal apoptotic rate of HT29, HCT116 and CaCo2 cells were not statistically different from each other. T84 cells had the lowest basal apoptosis rates in the absence of BuA.

Over the BuA concentration range tested, HT29 and SW480 responded to BuA in a dose dependent manner, with significant increases in apoptosis observed between 5mM and 20mM for HT29 cells. SW480 cells showed significant increases in apoptosis at 15mM-20mM BuA, with a strong dose dependent trend present for BuA concentrations ranging from 5mM-10mM. CaCo2, HCT116 and LIM1215 cells reached a maximal apoptotic response at the lowest concentration of BuA tested (2.5mM), and unlike the HT29 cells, plateaued rates of apoptosis were observed between 5mM and 20mM. Results from this experiment have been summarised in Table 3.3, which shows the percentage increase in apoptosis relative to no BuA treatment. At 20mM BuA, HCT116 cells responded with the largest increase in apoptosis, followed by LIM1215 cells and HT29 cells. CaCo2 cells underwent significantly less induction of apoptosis, while the SW480 cells responded with a small increase in apoptosis following BuA exposure. Furthermore, the CaCo2 cells showed significantly less apoptosis at 20mM compared with 5mM. T84 cells showed a slight trend of increased apoptosis with increasing BuA concentrations, although this was not significant at any concentration of BuA tested.

% increase in apoptosis relative to basal apoptosis (0mM BuA control)		
Cell line	**5mM**	**20mM**
HT29	277.4 ± 44.5	474.0 ± 33.4**
SW480	24.8 ± 10.9	36.1 ±7.0
HCT116	558.4 ± 41.9	571.9 ± 21.2
CaCo2	183.0 ± 28.2	110.4 ± 6.1*
LIM1215	377.2 ± 27.5	489.2 ± 45.0**
T84	16.0 ± 3.7	37.59 ± 7.9

Table 3.3: Percentage increase of apoptosis relative to 0mM BuA in each cell line after administration of 5mM and 20mM BuA. (*) $p<0.05$ (**) $p<0.01$ (***) $p<0.001$ denotes a significant difference from 5mM BuA for the same cell line (One-Way ANOVA with Tukey's Post Hoc testing using values from each cell line respectively).

3.4.3 Proliferation in HT29 and HCT116 cells in the presence of G-Protein inhibition

Proliferation of HT29 cells (Figure 3.3a and 3.3b) and HCT116 cells (Figure 3.4a and 3.4b) was decreased with the addition of BuA, consistent with the previous decrease observed in Figure 3.1. For both HT29 and HCT116 cells, GP2A did not alter the proliferation of cells in the absence of BuA at any concentration tested. For both HT29 and HCT116 cells, PTX did not alter the proliferative response of cells in the absence of BuA (0mM BuA). Furthermore, PTX appeared only to slightly enhance BuA-induced decreases in proliferation in both HT29 and HCT116 cells; however, this was not significant at any concentration of BuA or inhibitor. BuA's effects on proliferation were not altered by GP2A (5μM or 10μM) at any BuA concentration tested. In HCT116 cells, the addition of PTX or GP2A did not significantly alter the proliferative effect of BuA. However, GP2A showed a trend for a slight increased HCT116 cell proliferation in the presence and absence of BuA, but this trend was small and not significant. The decrease in proliferation following exposure to 5mM and 20mM BuA in the presence of GP2A and PTX are summarised in Table 3.4.

% decrease in proliferation relative to basal proliferation (0mM BuA control)		
HT29 Cells	**5mM**	**20mM**
BuA only (PTX)	27.5 ± 4.1	53.9 ± 2.2***
BuA + 500ng/μl PTX	28.2 ± 2.4	54.2 ± 1.0***
BuA + 1000ng/ml PTX	29.9 ± 2.6	53.5 ± 2.7***
BuA + 2000ng/ml PTX	37.8 ± 3.3	56.1 ± 5.7*
BuA only (GP2A)	33.7 ± 3.0	56.1 ± 3.3***
BuA + 5μM GP2A	31.5 ± 1.8	53.7 ± 1.5***
BuA + 10μM GP2A	28.5 ± 3.7	51.5 ± 2.6***
HCT116 Cells	**5mM**	**20mM**
BuA only (PTX)	39.9 ± 5.7	41.4 ± 7.1
BuA + 500ng/ml PTX	45.2 ±5.3	45.0 ± 6.2
BuA + 1000ng/ml PTX	46.5 ± 3.7	46.2 ± 5.0
BuA + 2000ng/ml PTX	51.6 ± 3.4	50.7 ± 4.0
BuA only (GP2A)	41.1 ± 7.3	45.9 ± 10.2
BuA + 5μM GP2A	31.4 ± 6.2	38.9 ± 8.1
BuA + 10μM GP2A	27.5 ± 5.1	33.5 ± 6.5

Table 3.4: Percentage decrease of proliferation relative to 0mM BuA in each cell line at 5mM and 20mM BuA, and varying concentrations of PTX and GP2A. (*) $p<0.05$ (**) $p<0.01$ (***) $p<0.001$ denotes significant differences from 5mM BuA for the same cell line. (#) $p<0.05$ (##) $p<0.01$ (###) $p<0.001$ denotes significant difference from the appropriate BuA only control. When testing the effects of G-protein inhibitors on BuA-induced changes to proliferation no concentrations of either PTX or GP2A were significantly different from the appropriate BuA only control. (One-Way ANOVA with Tukey's Post Hoc testing using all values generated from each cell line and G-protein inhibitor).

3.4.4 Apoptosis of HT29 and HCT116 cells in the presence of G-Protein inhibition

HT29 cells demonstrated an increase in apoptosis with BuA addition as seen previously in 3.2. PTX inhibited the effect of BuA on apoptosis in a dose dependent manner (Figure 3.5a). The effect of GP2A (5μM and 10μM) was very similar to that seen with PTX, with a trend showing a reduction in BuA-induced apoptosis at 5μM (Figure 3.5b). The inhibition of BuA-induced apoptosis by GP2A was significant at 10μM GP2A with the addition of BuA 5mM-20mM BuA (Figure 3.5b).

BuA-induced apoptosis in the HCT116 cells occurred as observed previously (Figure 3.2) and the effect of PTX and GP2A on BuA-induced apoptosis can be seen in Figure 3.6a and 3.6b. In the HCT116 cells, there was a trend for an increased BuA-induced apoptosis with PTX at the 2000ng/ml concentration. However, this was not significant and occurred in the both the presence and absence of BuA, indicating a non-specific induction of apoptosis by this G-protein inhibitor. No significant changes in BuA-induced

apoptosis occurred with the addition of GP2A at any concentration of inhibitor or BuA. The increases in apoptosis following exposure to 5mM and 20mM BuA in the presence of GP2A and PTX have been summarised in Table 3.5.

% increase in apoptosis relative to basal apoptosis (0mM BuA control)		
HT29 Cells	**5mM**	**20mM**
BuA only (PTX)	309.1 ± 26.6	551.3 ± 20.4 ***
BuA + 500ng/μl PTX	193.8 ± 17.2$^{###}$	414.6 ± 19.7$^{***/###}$
BuA + 1000ng/ml PTX	104.0 ± 18.8$^{###}$	285.1 ± 27.6$^{***/###}$
BuA + 2000ng/ml PTX	-6.1 ± 5.3$^{###}$	89.2 ± 14.6$^{*/###}$
BuA only (GP2A)	258.7 ± 51.7	474.0 ± 33.4**
BuA + 5μM GP2A	193.0 ± 41.7	378.1 ± 20.3*
BuA + 10μM GP2A	80.9 ± 29.6$^{#}$	380.1 ± 22.0$^{**/##}$
HCT116 Cells	**5mM**	**20mM**
BuA only (PTX)	469.1 ± 18.1	444.3 ± 43.5
BuA + 500ng/ml PTX	516.3 ± 44.6	509.7 ± 15.7
BuA + 1000ng/ml PTX	494.9 ± 46.7	493.0 ± 22.9
BuA + 2000ng/ml PTX	605.6 ± 39.3	622.3 ± 63.9
BuA only (GP2A)	463.1 ±25.7	519.8 ± 28.2
BuA + 5μM GP2A	503.2 ± 37.1	458.6 ± 28.4
BuA + 10μM GP2A	499.3 ± 37.6	413.2 ±28.2

Table 3.5: Percentage increase of apoptosis relative to 0mM BuA in HT29 and HCT116 cells line at 5mM and 20mM BuA, and varying concentrations of PTX and GP2A. (*) $p<0.05$ (**) $p<0.01$ (***) $p<0.001$ denotes significant difference from 5mM BuA for the same cell line. (#) $p<0.05$ (##) $p<0.01$ (###) $p<0.001$ denotes significant difference from the appropriate BuA only control. (One-Way ANOVA with Tukey's Post Hoc testing using all values generated from each cell line and G-protein inhibitor).

3.5 Discussion

Despite numerous studies which have provided substantial evidence that BuA exposure increases apoptosis and decreases proliferation *in vitro*, there are large variations in the approaches used to measure the effects of BuA. These variations make comparisons between cell lines difficult and in the present study a consistent approach was used across the cell lines tested to allow for comparisons. The current study established the proliferative and apoptotic response of HT29, SW480, HCT116, CaCo2, LIM1215 and T84 cells following BuA exposure using the Cell Titre Blue™ Cell Viability Assay (Figure 3.1) and Apo-One™ Homogenous Caspase 3/7 Assay (Figure 3.2).

3.5.1 Basal growth rates of 6 different CRC cells

The initial seeding density was consistent for all 6 CRC cell lines, which enabled any differences in basal growth rates to be detected. The fluorescence values for the proliferation and apoptosis in the CRC cell lines examined demonstrate that 4 of the 6 CRC cell lines have a higher basal proliferation rate compared with the remaining 2 cell lines (Figure 3.1). HCT116, LIM1215, HT29 and SW480 cells all have approximately the same rate of growth in the untreated 0mM BuA control groups, whereas CaCo2 and T84 cell growth is much slower. The growth rates determined for each cell line in this experiment are very similar to the growth rates (as indicated by splits for maintenance culture) recorded in Table 3.1.

3.5.2 Response to BuA yields different patterns of response across CRC cell lines

The current study shows that each cell line has a characteristic response to BuA exposure as evident by the results displayed in Figure 3.1 and 3.2. Following BuA treatment the following patterns of response across CRC cell lines were observed:

1. A consistent and strong increase in apoptosis and decrease in proliferation observed in proliferation displayed by HT29 and HCT116 cells.
2. A small or weak response in apoptosis and proliferation displayed by CaCo2 cells.
3. A strong apoptotic response and weak decrease in proliferation observed in LIM1215 cells.
4. A high level of basal apoptosis and moderate BuA-induced decrease in proliferation and increase in apoptosis displayed by SW480 cells.
5. A resistance to BuA was observed in T84 cells.

Many groups have also reported differences in the magnitude of BuA effects in different CRC cell lines. Comalada et al (Comalada et al., 2006) investigated the 'normal' colon epithelial cell line FHC and adenocarcinoma HT29 cells and observed that the response of CRC cell lines to BuA exposure was dependent on the cellular phenotype. BuA significantly decreased the proliferation in the undifferentiated and highly proliferative HT29 cells, but had no effect on the normal cell line. From these results, Comalada et al concluded that "although butyrate could exert anti-proliferative effects in tumour progression, its production is safe and without consequences for the normal epithelium growth". Kautenburger et al (Kautenburger et al., 2005) demonstrated that BuA suppressed cell growth to a greater extent in the adenoma cell line LT97 compared to

the adenocarcinoma cell line HT29, which suggests that BuA may be more effective in early stage cancer compared to later stages. Hague et al (Hague et al., 1995) measured apoptosis in 3 adenoma and 4 carcinoma cell lines and showed that 2 of the 4 carcinoma cell lines were more resistant to BuA-induced apoptosis and concluded that the escape from induction of apoptosis may be important in the progression of cancer.

Bonnotte et al (Bonnotte et al., 1998) demonstrated that CaCo2 cells were resistant to Fas and BuA mediated apoptosis, and also showed that in 7 other CRC cell lines tested there was a differential induction of apoptosis following exposure to BuA and Fas-ligand. In 1993, Hague et al (Hague et al., 1993) investigated the apoptotic response of 6 adenoma cell lines, 3 clones of the adenoma cell lines and 7 carcinoma cell lines. While this study was designed to show that BuA-induced apoptosis is p53 independent, the results from this publication can also be used to demonstrate that the extent of apoptosis induced by BuA varies depending on the cell line investigated, as each of the cell lines had varying responses to BuA. In 1999, Stokrova et al (Stokrova et al., 1999) showed that the magnitude of differentiation in CRC cells was dependent on the cell line tested. Furthermore, in 2006 Stokrova et al (Stokrova et al., 2006) demonstrated that although 4 cell lines cloned from parent HT29 cells could all undergo differentiation in response to BuA, this occurred to vastly different extents. Each of these papers shows that the response of different CRC cell lines to BuA exposure is highly variable, and depends on the cell line examined. A similar result was also observed in the current study, with CaCo2 cells being somewhat resistant and T84 cell lines almost completely resistant to BuA-induced apoptosis, whereas HCT116 cells were extremely sensitive and recorded a 571 ± 21% increase in apoptosis following 20mM BuA exposure (Table 3.2 and 3.3). Collectively, these findings show that the magnitudes of the apoptotic and proliferative responses to BuA are cell line specific.

3.5.3 Apoptosis and Necrosis

It was of interest to note that CaCo2 cells showed significantly less apoptosis at 20mM compared with 5mM BuA treatment (Table 3.3). At the higher concentration of BuA, it is possible that the CaCo2 cells have undergone apoptosis, and are now entering late stage apoptosis or have become necrotic. If the CaCo2 cells are largely necrotic at the 20mM BuA concentration, significantly less caspase 3 and 7 would be present, and therefore could account for the lower level of apoptosis observed using an assay that measures caspases as a marker of apoptosis. Other studies have demonstrated that following BuA exposure mouse colon cancer cells can be categorised into early stage

apoptosis, late stage apoptosis and necrosis (Fan et al., 1999). Hague et al (Hague et al., 1993) have demonstrated that amongst CRC cells that are floating in culture, the percentage of those cells that are apoptotic can vary from 36% to 96% depending on the cell line tested, indicating that the remaining percentage of cells are necrotic rather than apoptotic. To more comprehensively investigate this finding, future experimentation could include the use of techniques to enable apoptosis and necrosis to be measured in the same assay, such as FACS analysis with Annexin V and Propriduim Iodide staining as described in Allen et al (Allen et al., 1997).

3.5.4 T84 cells appear resistant to BuA-induced apoptosis

Interestingly, no significant effect on apoptosis was observed with T84 cell lines following exposure to BuA, despite the other cell lines displaying significant BuA-mediated increases in apoptosis (Figure 3.2). This result may appear unexpected, but extensive literature searches identified no studies describing the proliferative and apoptotic response of T84 cells following BuA exposure. Other examples of T84 resistance to apoptosis inducing agents have, however, been reported. Investigation of inhibitors of epidermal growth factor receptor (EGF-R) dependent signalling by the tyrphostin A25 (a protein tyrosine kinase inhibitor), was shown to induce apoptosis in HT29/HI1 and SW480 CRC cell lines, but to a much lesser extent in T84 cells (Partik et al., 1999). When the same investigators measured DNA synthesis, HT29 and SW480 cells showed efficient decreases following exposure to A25 whereas the T84 cells did not. When the same group tested another tyrphostin, AG1478, they demonstrated efficient inhibition of DNA synthesis and induction of apoptosis in all 3 cell lines. This example demonstrates a differential response of T84 cells, including one case of resistance, to the apoptotic agents A25 and AG1478. This would suggest that the apparent resistance of T84 cells to BuA-induced apoptosis may be a cell line specific effect as other apoptotic inducing agents produce a similar effect in these cells.

In a publication by Hague et al (Hague et al., 1995) 2 of 4 carcinoma CRC cell lines investigated were somewhat resistant to BuA-induced apoptosis, as was seen in the T84 cell lines in the current experiments. Bonnotte et al (Bonnotte et al., 1998) also observed BuA resistance in CaCo2 cells when they investigated cancer cell sensitisation to Fas-mediated BuA-induced apoptosis in 8 different CRC cell lines. Fas is a protein receptor that, upon ligand stimulation or receptor cross linking, can induce apoptosis (Nagata, 1994; Nagata and Golstein, 1995). In the study by Bonnotte et al (Bonnotte et al., 1998), HT29, HRT18, HCT15, HCT-8R, SW480, SW620 and rat colon cancer cell

line, PROb, all showed an increase in apoptosis following the addition of BuA and Fas ligand, but CaCo2 cells did not. It is possible that the T84 cells in the current experiment, like the CaCo2 cells in the Bonnotte et al study (Bonnotte et al., 1998) are largely resistant to BuA-induced apoptosis.

Other instances of CRC cell resistance to BuA exposure have been observed. Mariadason et al (Mariadason et al., 2001) demonstrated that BuA-induced apoptosis, cell cycle arrest, differentiation and other effects could occur in undifferentiated CaCo2 cells, however, in spontaneously differentiated CaCo2 cells the author notes that the cells are 'essentially resistant' to the effects of BuA. Lu et al (Lu et al., 2005) and Halttunen et al (Halttunen et al., 1996) have reported that T84 cells are highly differentiated and Niv et al (Niv et al., 1992) have shown that T84 cells also spontaneously differentiate in culture. Therefore, it is possible that the T84 cells grown for these experiments were predominantly differentiated and were consequently not responsive to BuA. Some cells that are grown to confluence or past confluence can undergo spontaneous differentiation (Mariadason et al., 2000b). It is possible that the cells were differentiated prior to the arrival in this laboratory. To confirm the T84 results in this Chapter, it would be of interest to access T84 cells of different origins to test the differentiation status of the cell lines stocks using a differentiation marker such as alkaline phosphatase, then re-testing the apoptotic and proliferative response following BuA exposure.

3.5.5 Different CRC cell lines have a unique pattern of response to BuA

The pattern of response to BuA is also unique to the particular cell line. HT29 and SW480 cells responded in a dose-dependent manner when proliferation was investigated across the BuA concentrations tested (Figure 3.1). When apoptosis was investigated, HT29, SW480 and LIM1215 cells responded in a dose-dependent manner (Figure 3.2). In contrast to this, CaCo2 and HCT116 cells reached maximum increases in apoptosis and decreases in proliferation at low concentrations of BuA, and maintained this magnitude of response for BuA concentrations up to 20mM (Figure 3.1 and 3.2). The different response of cell lines to BuA exposure does not appear to correlate with the progression of disease. However, it is difficult to use cell lines to produce definitive conclusions about disease staging due to inherent differences in the genetic make-up of each cell line as well as differences in the morphology and growth rates as seen in the Table 3.1.

3.5.6 BuA-induced changes: relationship between proliferation and apoptosis

BuA's actions include cell cycle arrest, decreased proliferation, increased terminal differentiation, and the induction of apoptosis. If each of these pathways were linked in a common effector/response relationship, it would be expected that the response of each cell line to BuA exposure would be similar when measuring apoptosis and proliferation. It is evident from the results presented in this Chapter that in some cell lines there is a disassociation of the apoptotic and proliferative relationship. For example, LIM1215 cells have one of the smallest changes in proliferation following BuA exposure, with only an 18 ± 5% and 26 ± 3% (Table 3.2) decrease in proliferation at the 5mM and 20mM BuA respectively. However, these cells have the second highest increase in apoptosis at 5mM and 20mM BuA of 377 ± 28% and 489 ± 45 respectively (Table 3.3). In CaCo2 cells, which have the second highest decrease in proliferation at 5mM BuA (37 ± 1%), the apoptotic response is small compared to some of the other cell lines (183 ± 28%) (Table 3.3). In HT29 cells treated with BuA, a similar disassociation of apoptosis and proliferation has been reported following investigation of the MCT1 transporter. RNAi knockdown studies (Cuff et al., 2005) demonstrated that the MCT1 transporter affects BuA uptake into the cell and BuA-induced changes on cell proliferation and differentiation, but MCT1 knockdown did not affect BuA-induced apoptosis.

Litvak et al (Litvak et al., 1998) have demonstrated links between cell cycle regulation, differentiation and apoptosis, and Heerdt et al (Heerdt et al., 1994) have suggested a link between pathways of proliferation, differentiation, apoptosis, and cellular shedding. Normal colonocytes *in vivo* are arranged in crypts with rapidly proliferating stem cells located deep within the crypt. Migration of these cells occurs from the base of the crypt to the luminal surface (Figure 1.1) (Potten and Morris, 1988). During the migration, the cells differentiate until the terminally differentiated epithelial cells near the top of the crypt are sloughed off into the colonic lumen (Gordon et al., 1992) (Orchel et al., 2005). Morphological studies show apoptotic cells amongst the differentiated cells near the crypt-lumen interface (Potten and Allen, 1977).

Interconnections between cell apoptosis and proliferation have been demonstrated by Alenzi et al (Alenzi, 2004) who provided several lines of evidence in their review to show an association between the two cellular functions. Some of the key genes and pathways that regulate apoptosis and proliferation are common to both, and Evan et al (Evan et al., 1992) demonstrated that the c-myc protein, which could stimulate

proliferation, can also induce apoptosis when the availability of growth factors was limited. Furthermore, Gil-Gomez et al (Gil-Gomez et al., 1998) demonstrated that Bax and Bcl-2, genes involved in the regulation of apoptosis, can modulate $p27^{Kip1}$, a Cdk inhibitor, which in turn regulates the cell cycle regulatory protein Cdk2. This finding suggests a link between apoptosis and cell cycle regulation and supports the results obtained in this Chapter for cell lines such as HCT116 cells; which respond to BuA with the largest changes in proliferation at 5mM, 47 ± 7% (Table 3.2) and apoptosis 558 ± 42% (Table 3.3).

In contrast, there is evidence to show that the relationships between apoptosis, proliferation and differentiation are not exclusively linked. Although many genes are common to apoptosis, proliferation and cell cycle regulation, some of these genes are capable of interfering with one of these processes without affecting the others. An example is the tumour suppressor gene p53, which can induce both cell cycle arrest and apoptosis, and can have separate effects on these two pathways (Miyashita and Reed, 1995). When p53 is restored in cell lines which lack p53 expression, the cell lines undergo predicted cell cycle arrest and apoptosis. However, in the presence of the apoptosis blocking protein Bcl-2, p53-induced apoptosis is blocked, but p53 cell cycle arrest is not altered (Selvakumaran et al., 1994; Wang et al., 1993). Orchel et al (Orchel et al., 2005) observed a similar disassociation between differentiation and apoptosis. In the current study, 1mM BuA-induced the greatest increase in differentiation in HT29 and CaCo2 cells, with no significant increase in apoptosis. The largest increase in apoptosis was observed with exposure to 10mM BuA, suggesting that the biochemical pathways that induce differentiation and apoptosis are unique and not simply connected. Collectively the results of the experiments described in this Chapter and the research of others suggests that in some cell lines apoptosis and proliferation can occur together indicating both pathways are possibly linked, however, in other cells there appears to be no association between the two pathways.

3.5.7 Activation of GPCRs is a possible mechanism for BuA-mediated changes to apoptosis and proliferation

Although many reports have focused on BuA-induced decreases in proliferation and increases in differentiation and apoptosis, the exact mechanism(s) by which BuA initiates these outcomes remains unknown. Abnormalities in GPCRs have been linked to a number of pathological diseases, including colon adenomas and carcinomas (Horn et al., 2000) (Heasley, 2001). As GPCR40-43 were recently shown to be activated by

BuA and other SCFAs and FAs, G-protein function was examined in the current study in relation to BuA-induced changes to apoptosis and proliferation. The activation of GPCR41 and 43 in response to ligand binding was shown to be inhibited by PTX (Le Poul et al., 2003) (Brown et al., 2003), and it was therefore of interest to observe and to determine whether BuA-induced changes to apoptosis and proliferation were G-protein mediated. As described in Chapter 1, GPCR43 has been reported to activate both the $\alpha_{i/o}$ and α_q subunit, therefore the α_q specific inhibitor GP2A (McKillop et al., 1999) was also examined.

3.5.8 BuA-induced changes to proliferation are not modulated by G-protein inhibitors

In HT29 and HCT116 cells exposed to PTX a trend indicated that increasing concentrations of PTX led to a decrease in cell proliferation. However, this did not achieve significance at any concentration of BuA or PTX (Figure 3.3a) in the HT29 cells. The trend was also observed in the absence of BuA (0mM) which suggests that the PTX may have an effect on the proliferation of HT29 cells, and that this effect is enhanced with the addition of BuA. In HCT116 cells, no changes in BuA-induced decreases in proliferation were observed in the presence of PTX at 500ng/ml or 1000ng/ml. At a concentration of 2000ng/ml PTX a trend of increased proliferation occurred at all concentrations of BuA tested, although this was not significant when tested using One-Way ANOVA with Tukey's Post Hoc testing except at 2000ng/ml PTX in the absence of BuA (Figure 3.6a). As PTX induces an increased proliferation level in the absence of BuA, the results indicated the possibility that the observable (but not significant) increase in apoptosis in response to 2000ng/ml PTX and 1-20mM BuA exposure is a non specific effect that is not related to BuA-induced apoptosis. GP2A had no significant effect at any concentration of inhibitor or BuA in either cell line (Figure 3.5b and 3.6b). A small trend of increased proliferation of HCT116 cells exposed to GP2A was observed (Figure 3.4b) and occurred in the absence of BuA, suggesting that the small (but not significant) effect of GP2A is BuA independent. The results from this section suggest that BuA-induced decreases in proliferation are not modulated by G-protein mechanisms in either HT29 or HCT116 cells.

3.5.9 BuA-induced changes to apoptosis in HT29 cells but not HCT116 cells are modulated by G-protein inhibitors

In HT29 cells, (Figure 3.5a and 3.5b) but not HCT116 cells (Figure 3.6a and 3.6b), PTX and GP2A consistently inhibited BuA-induced apoptosis. These results suggest that G-

protein inhibitors, and thus GPCRs have the potential to modulate BuA-induced apoptosis in HT29 cells, but do not significantly influence BuA-mediated apoptosis in HCT116 cells. This is an interesting finding, and suggests at least 2 possibilities: (1) that apoptosis could potentially be occurring through different pathways in these two different cell lines or (2) that G-proteins regulate BuA-induced apoptosis in HT29 cells and that this regulation is absent or lost in HCT116 cells.

PTX and GP2A act through different α-subunits, therefore, the results from these experiments indicate that apoptosis in HT29 cells can occur through either an $\alpha_{i/o}$ subunit or an α_q subunit pathway (Figure 1.8). As PTX blocks the $\alpha_{i/o}$ subunit, it can be expected that the usual activation of adenylyl cyclase and subsequent inhibition of cAMP is also blocked. In the absence of PTX, ligand binding to the GPCR would induce an inhibition of cAMP leading to a secondary messenger cascade as summarised in Figure 1.8. The α_q subunit activates PLC, leading to the activation of a different secondary messenger cascade, also summarised in Figure 1.8. This effect is blocked by the addition of GP2A. The result from the current study may imply that BuA-induced apoptosis can be modulated through both cAMP and PLC pathways in HT29, but not HCT116 cells. Similar results have also linked apoptosis to cAMP (Lin and Ye, 2003) and PLC pathways (Turner et al., 2000) in different cell types, and BuA exposure has also been linked to decreased Cl^- excretion in T84 CRC cells, arising from decreased cAMP (Resta-Lenert et al., 2001). The same investigators also demonstrated a decrease in the expression and activity of adenylyl cyclase in T84 cells following BuA exposure.

As previously described in Chapter 1, other investigators have shown collectively that many of the effects induced by BuA are G-protein mediated. Davis et al (Davis et al., 2000) demonstrated that PTX could block BuA-induced differentiation in the K562 leukaemia cell line, and this group also demonstrated a significant increase in both the mRNA and protein expression of G_i, G_q, and G_β α-subunits following exposure to BuA. Additionally, Senga et al (Senga et al., 2003) demonstrated that GPCR43 expression was strongly induced in HL-60 and U937 leukaemia cells during their differentiation to monocytes. Since the commencement of the experiments in this thesis it has been shown that in rat adipose tissue, that RNAi knockdown of GPCR43 rendered the cells unable to undergo adipose differentiation in response to acetate and propionate (Hong et al., 2005). Furthermore, GPCR41 has been implicated in apoptosis in the rat myocardium cell line H9c2 following induced hypoxia (Kimura et al., 2001). Interestingly

in the MCF-7 human breast cancer cell line, acetate, propionate and BuA-induced an increase in intracellular Ca^{++}, which was not blocked with the addition of PTX (Yonezawa et al., 2006). Yet, when the same group used RNAi knockdown of GPCR43 they demonstrated a significant inhibition of the propionate-induced increase in intracellular Ca^{++}. Although, there are many examples of apoptosis being mediated by G-proteins, it must be acknowledged that this is not the case for all cell types which is evident in this Chapter by the inconsistent effects of PTX and GP2A on two different CRC cell lines. Collectively, the findings in this Chapter suggests that apoptosis is modulated by G-protein inhibitors in some cases and that this finding shares a growing list of cellular processes mediated by BuA, some of which have also been reported to be modulated by this inhibition.

3.5.10 Summary

There are at least 3 conclusions which can be made from the experiments conducted in this Chapter:

1. BuA is effective in reducing proliferation and enhancing apoptosis in 5 of 6 CRC cell lines.
2. With regard to a direct linkage between enhanced apoptosis occurring with decreased proliferation, the results in this Chapter indicate that this is not a constant and 2 outcomes may occur.
    a. An apparent association between proliferation and apoptosis (eg HT29, SW480 and HCT116 cells).
    b. Either no association or a loose association between proliferation and apoptosis (eg LIM1215, CaCo2 or HT29 cells in the presence of PTX and GP2A).
3. The modulation of apoptosis but not proliferation in HT29 cells by PTX or GP2A suggests a role of G-proteins in modulating apoptosis in response to BuA in the cells.

The results from this Chapter form the questions addressed in the following Chapter. If G-proteins are involved in BuA-induced apoptosis in HT29 cells, is GPCR43 responsible for mediating this event? To examine this question, the magnitude of the expression of GPCR43 was investigated in the 6 CRC lines used in the current Chapter.

4 Identification of GPCR43 on colorectal cancer cell lines by RT-PCR

4.1 Introduction

The previous chapter has confirmed that BuA can induce decreased proliferation and increased apoptosis in CRC lines to different degrees in 6 different CRC cell lines. Accumulated evidence strongly suggests that the effects of BuA are largely mediated through interactions with histone deacetylase (Candido et al., 1978) (Wu et al., 2001). As described in Chapter 1 (section 1.8.5), BuA inhibits histone deacetylase leading to hyperacetylation and subsequent "unwinding" of the histones. This results in an increased transcription of genes which regulate cell cycle and apoptosis. Although histone involvement has been demonstrated, the exact mechanisms and pathways involved in this process remain largely undetermined.

It has been postulated that extracellular BuA is actively transported into cells by cell surface proteins such as MCT1 and SLC5A8, and that the regulation of these transporters may play a role in exerting BuA-induced effects. Additionally, it was hypothesised in the current study that a family of GPCRs (GPCR40-43), which were described as binding FAs and SCFAs, may be involved in mediating BuA-induced increases in apoptosis and decreases in proliferation. As discussed in Chapter 1, although an entire family of GPCRs were described as binding SCFAs, only GPCR43 was investigated in this project as a possible BuA receptor. At the time of commencement of this study, GPCR43 was shown to be expressed predominantly on immune cells, in particular monocytes, neutrophils, PBMC cells (Brown et al., 2003) (Le Poul et al., 2003) and spleen cells (Le Poul et al., 2003). In the previous Chapter it was shown that G-protein inhibition altered BuA's influence on apoptosis in HT29 cells (but not HCT116 cells). The focus of the experiments in this chapter was to determine if GPCR43 exists on CRC cells, which had not been previously reported when these experiments were conducted. With the differences in results from the G-protein inhibition experiments in the previous chapter, it was of interest to determine if GPCR43 is present on all CRC cells examined.

4.2 Approach

To assess the expression of GPCR43 on 6 CRC cell lines using RT-PCR.

4.3 Methods

4.3.1 Ethics

Ethical approval for the work conducted on human samples (PBMCs) in this experimentation was obtained from the CSIRO human ethics committee.

4.3.2 Molecular biology techniques

A detailed description of the experimentation conducted in this Chapter has been provided in the Material and methods section. Refer to section 2.2.2-2.3.3 for methodology.

4.4 Results

4.4.1 PCR

Total cellular RNA was extracted successfully as indicated by strong bands observed at 1.1kb and 2.7kb on a 1% agarose gel, which are representative of the 18S and 28S ribosomal bands (Figure 4.1). This demonstrates the quality and integrity of the RNA from each of the extractions. The ratio of the optical density (OD) at 260nm divided by absorbance at 280nm ($Ab_{260}/_{280}$) gave values of 1.8 or higher for all samples, which also indicated isolated RNA of high quality.

GPCR43 expression, including the positive and negative controls, was simultaneously optimised on U937 monocytic cells and in human PBMC cells prior to the application of this methodology to the CRC cell lines (Figure 4.2). Controls containing no template were also used routinely throughout the experimentation to monitor for reagent contamination.

GPCR43 lacks introns, and therefore an mRNA transcript will have the identical sequence to the gene, and any contaminating gDNA in the RNA extracts would lead to false positive errors. To ensure all gDNA had been removed from each of the RNA samples, non transcribed RNA was added to a PCR reaction, run on an agarose gel, then stained with ethidium bromide. The absence of detectable bands indicated the RNA was gDNA free (Figure 4.3 for CRC cell lines Figure 4.2a for U937 and 4.2b for PBMC).

Once the RNA sample was confirmed to be gDNA free, cDNA was generated and used as the template for further PCR amplification reactions. The PCR was initially conducted using primers to amplify the housekeeping gene β-Actin to ensure cDNA synthesis had occurred correctly and evenly across all samples (Figure 4.4 for CRC cells Figure 4.2a for U937 and 4.3b for PBMC). Quantitative densitometry of each band was conducted using gel image analysis software Scion Image (Figure 4.4b). Another gene, MCT1, was also investigated and shown to be readily amplified in each cell line (Figure 4.7).

The PCR for GPCR43 used 2 primer combinations to give amplicons of 330bp and a full length fragment of 993bp. The conditions were optimised using the U937 cell line and PBMC cells (Figure 4.2) then applied to the 6 CRC cell lines (Figure 4.5a, 4.5b). Three of the 6 cell lines, HT29, CaCo2 and T84, showed expression of GPCR43 when using the 330bp primers, and 2 of the 6 cell lines, HT29 and T84 cells, showed expression when using the 993bp primers. Quantitative densitometry values for all of the gel images are shown in Figure 4.5c, 4.5d respectively.

To determine the presence or absence of the gene in all cell lines gDNA was extracted from all 6 cell lines and tested for GPCR43. The results indicated that each of the 6 CRC cell lines have a copy of the gene coding for GPCR43 (Figure 4.6).

4.5 Discussion

4.5.1 RNA extractions : yield, quality, and purity

RNA quality is of the upmost importance when analysing mRNA expression to avoid false results for differences in expression levels due to RNA degradation (Bustin et al., 2005; Bustin and Nolan, 2004a; Kubista et al., 2006). Determining RNA quality can be achieved by inspection of 18S and 28S ribosomal bands using gel electrophoresis and by calculation of $Ab_{260/280}$ (Manchester, 1996).

In the current study, RNA quality and quantity was confirmed using both inspection of 18S and 28S rRNA bands using gel electrophoresis and spectrophotometry using NanoDrop technology. The $Ab_{260/280}$ should be greater than 1.8 if the quality is to be considered acceptable (Fleige and Pfaffl, 2006). However, this technique alone has been criticised for only assessing RNA quality relative to protein contamination, and not gDNA contamination. Bustin and Nolan (Bustin and Nolan, 2004b) have demonstrated

that the OD values can be altered by any contaminating gDNA, leading to an overestimation of RNA concentration. However, as mentioned in the Results section, the complete removal of gDNA was paramount, as GPCR43 does not contain introns and any contaminating gDNA would lead to false positive results. In this study detectable gDNA was removed by double DNase treatment (Figure 4.3) and ensured that incorrect OD calculations arising from contaminating gDNA were not a factor in this study. Considerable effort was also required to elucidate an RNA extraction technique that yielded RNA with no detectable gDNA. In every extraction the RNA isolated from all sources was of high quality, indicated by strong 18S and 28S bands observed using gel electrophoresis in Figure 4.1 and $Ab_{260/280}$ values of greater than 1.8.

4.5.2 Uniform expression of the β-Actin housekeeping gene

β-Actin is a commonly used housekeeping gene (de Kok et al., 2005; Rubie et al., 2005) and results from this study show uniform expression from the 6 CRC cell lines (Figure 4.4) as well as strong expression in both PBMC and U937 cells (Figure 4.2a and 4.2b). Although the concept of a gene that is constitutively expressed across all cell types in all conditions will be discussed in the next Chapter, the β-Actin housekeeping gene was used in these experiments to confirm that cDNA synthesis had occurred in an even and reproducible manner across all cell lines, thus acting as a RNA 'loading control'. This is based on the principle that if equal amounts of RNA from each of the CRC cell lines were added to the reverse transcription (RT) step, the intensity of the β-Actin amplicons generated from the PCR should be approximately equal.

In the current study, bands of approximately equal intensity are observed, indicating a successful RNA loading and cDNA synthesis (Figure 4.4a). The quantitative densitometry analysis in Figure 4.4b confirmed approximately equivalent intensities across the cell lines. While some fluctuations were observed in the intensity of β-Actin bands across the 6 CRC cell lines, these were minor, particularly when compared to changes observed in GPCR43 (Figure 4.5a, 4.5b, 4.5c and 4.5d). For the purpose of the current study, the consistency in β-Actin shows that RNA loading was even and cDNA synthesis occurred uniformly, suggesting that the differences in GPCR43 expression observed across cells lines was genuine.

4.5.3 Expression of GPCR43 is observed in 3 of 6 CRC cell lines tested

Results from the U937 cell line and PBMC cells demonstrated that the GPCR43 receptor is present in these cell types (Figure 4.2a and 4.2b), as observed by the

amplification of both the 330bp and 993bp amplicons. This finding confirms the results found by Le Poul et al (Le Poul et al., 2003) and Brown et al (Brown et al., 2003). It also allowed for the optimisation of cycling conditions and reagent concentrations. The controls for this experiment, including housekeeping gene amplification, were satisfactory in all cases and the “no template controls” were blank as predicted.

When the same reaction was conducted in the 6 CRC cell lines only 3 cell lines, HT29, CaCo2 and T84 cells, but not SW480, LIM1215 or HCT116 cells, showed expression of the receptor when using primers to generate 330bp amplicons (Figure 4.5a). When tested using the primers to amplify 993bp amplicons, expression could only be detected in HT29 and T84 cells (Figure 4.5b). In both cases, HT29 cells showed strong expression, indicated by bright bands, whereas expression from T84 and CaCo2 cells was very weak or not present (CaCo2, 993bp). The quantitative analysis using band intensity is also shown in Figure 4.5c and 4.5d. All reactions consisted of 40 PCR cycles, which is the maximum cycle number that should be used to prevent non-specific amplification (Fryer et al., 2002), therefore these results indicate low levels of expression in T84 and CaCo2 cells.

The lack of a band for the CaCo2 cells in the 993bp reaction when it was present in the 330bp reaction is likely to be due to inefficient priming. To design primers for amplification of the full length of the gene, the placement of the primers is limited to the beginning and end of the mRNA transcript. Although every effort was made to construct the primers within well documented guidelines, such as G-C content, melting temperatures (T_m), primer length and others (Dieffenbach et al., 1993), the primer design was not ideal because of the restrictive placement. Primers to amplify the full length sequence registered many issues when tested in primer design softwares programs (NetPrimer and Primer Express) including primer dimers and hairpin loops. Verification of this absence of expression in CaCo2 cells can be tested by using a more sensitive measure of mRNA, such as Quantitative Real Time PCR (Q-PCR), as was conducted in Chapter 5.

The results from this Chapter suggest expression of GPCR43 in HT29, CaCo2, T84 cells but not HCT116, LIM1215 or SW480 cells. It is interesting that only 3 of the 6 cell lines express this receptor, therefore a gDNA extraction, and subsequent PCR probing for the GPCR43 gene was conducted to ensure no gene deletion or other type of mutation leading to loss of the gene had occurred. Gene mutations are not uncommon in cultured cell lines, for example Bryan et al (Bryan et al., 2002) demonstrated loss of

the EP300 gene was common in colorectal cancer cell lines. However, in the current study all 6 of the CRC cell lines have a copy of the GPCR43 gene, as shown by the amplification of GPCR43 from extracted gDNA (Figure 4.6). This result suggests that the cell lines that do not express GPCR43 do not require expression of this gene, and it is possible these cell lines have a faulty transcription mechanism and, therefore, an inability to express the gene.

The expression bands for GPCR43 in CaCo2 and T84 cells were of low intensity, and indicated low levels of expression. The cDNA quality was tested using an unrelated non-housekeeping gene, MCT1. It can be seen in Figure 4.7 that MCT1 can readily be amplified using the cDNA from all 6 cell lines, and provides further evidence to confirm the integrity of the cDNA.

4.5.4 Is GPCR43 expression required for BuA-induced increases in apoptosis and decreased proliferation?

The results of the studies described in this and the previous Chapter have been summarised in Table 4.1.

	BuA		PTX		GP2A		GPCR43 expression
Cell Line	**Apoptosis**	**Proliferation**	**Apoptosis**	**Proliferation**	**Apoptosis**	**Proliferation**	
HT29	↑	↓	inhibited by PTX	unchanged by PTX	inhibited by GP2A	unchanged by PTX	√
SW480	↔	↓					x
HCT116	↑	↓	unchanged by PTX	unchanged by PTX	unchanged by PTX	unchanged by PTX	x
CaCo2	↑	↓					√
LIM1215	↑	↓					x
T84	↔	↔					√

Table 4.1: BuA-induced proliferative and apoptotic responses of 6 different CRC cell lines in the presence of BuA alone, PTX and GP2A and the expression of GPCR43 on each of the CRC cell lines. .(↑ indicates an increase in apoptosis, ↓ indicates a decrease in proliferation and ↔ indicates no change). Cell lines with receptor expression have been marked with √.

Table 4.1 shows that:

1. BuA can exert an effect on proliferation and apoptosis in cell lines in the absence of GPCR43 as detected by RT-PCR.
2. In HT29 cells the exposure to G-protein inhibitors can inhibit BuA-induced apoptosis in the presence of GPCR43.

The conclusions are qualified by the following:

1. There is a high dependence on the accuracy of RT-PCR alone to determine the presence or absence of GPCR43. While multiple controls were included in the experimental design, the possibility exists that GPCR43 may have been present but not detected.
2. Although mutations in key pro-apoptotic genes have been demonstrated to significantly alter the way CRC cell lines undergo apoptosis or proliferation, BuA-induced changes to both apoptosis and proliferation have been shown to proceed even in the absence of key pro-apoptotic and anti-proliferative genes such as p53 or p21 (Kim et al., 2004; Mahyar-Roemer and Roemer, 2001; Violette et al., 2002). Mahyar-Roemer et al (Mahyar-Roemer and Roemer, 2001) investigated the induction of apoptosis by BuA and 5 other anticancer agents in HCT116 cells which were HCT116 p53 wild type (p53 +/+), p53 homozygous negative (p53 -/-) or p21 homozygous negative (p21-/-). They demonstrated that the apoptotic response was significantly altered depending on the genotype. A similar result was observed by Archer et al (Archer et al., 1998b) who demonstrated the critical importance of p21 in BuA-induced inhibition of proliferation by stably transfecting the p21 gene into HT29 cells. Over-expression of p21 in the HT29 cell line led to a substantial decrease in cell growth. Furthermore, this group showed that in HCT116 p21 -/- cell lines, exposure to BuA did not induce decreases in cell growth, but in HCT116 p21 +/+ cells BuA significantly inhibited proliferation. Liu and Bodmer (Liu and Bodmer, 2006) analysed p53 mutations in 56 different CRC cell lines and demonstrated that 13 had functional p53, 21 had missense mutations, 18 had truncated p53 protein and 4 cell lines had no p53 transcripts. If the expression of the p53 and p21 genes or other genes varies in each of the 6 CRC cell lines tested in the current project, it may account for the differences in response to BuA observed in the 6 CRC cell lines tested in the previous Chapter. It will also make direct comparisons between the responses of each cell line to BuA complicated and difficult to analyse.

4.5.5 Summary

The final conclusion from the results in these two chapters is that GPCR43 may not be exclusively required for the BuA-induced decreases in proliferation and increased apoptosis, but may have a mediating role in some circumstances, particularly in the HT29 cell line. This conclusion is highly dependent on the reliability of the RT-PCR

technique. To investigate the role of this receptor with more certainty, specific knockdown or knockout techniques are required. It was also important to develop a more accurate and quantitative measure of GPCR43 expression in CRC cell lines so that knockdown can be measured. Q-PCR is an ideal assay as it can address the issue of sensitivity and can also be used for quantitation, and was therefore investigated in the following Chapter.

5 Further evidence for the presence of GPCR43 on colorectal cancer cell lines using Q-PCR

5.1 Introduction

A potential role for GPCRs in BuA-induced apoptosis was demonstrated using non-specific G-protein inhibitors described in Chapter 3 in HT29 cells, but not HCT116 cells. Results described in Chapter 4 showed that HT29 cells were positive for GPCR43 expression whereas the HCT116 cells did not show expression. This result provides some evidence that G-proteins may have a role in mediating BuA-induced apoptosis in selected CRC cell lines and implicates GPCR43 as a possible mediator. However, this conclusion is dependent on Q-PCR measurement of GPCR43. To further define the relationship between cell type and GPCR43 expression, Q-PCR was used.

Much debate surrounds the best approach to use in Q-PCR experiments due to a variety of methods available and multiple options to analyse the results. Consequently, there is no single standard procedure with which to conduct Q-PCR experiments and substantial optimisation and verification of methodology is required. The aim of this Chapter was to identify which Q-PCR techniques and analysis systems were most appropriate to test the expression of GPCR43 in different cell lines. Once established, the optimised Q-PCR assay was used to quantitatively investigate the expression of GPCR43 on the 6 CRC cell lines as previously analysed. The specific design of the experiments involving Q-PCR is critical and key aspects of the technology used in the experiments described in this Chapter follows.

5.2 Approach

5.2.1 Optimisation and verification of Q-PCR

Many of the issues that can affect the results obtained from Q-PCR experiments must be considered prior to conducting the actual PCR reactions. This includes factors such as the reverse transcription (RT) conversion of RNA to cDNA, the type of fluorescent probes to be used in the Q-PCR reaction and the quantitation or normalisation used. By controlling these factors it is possible to minimise the variability that can occur between Q-PCR reactions and these factors are discussed in more detail below.

The reliability of a single Q-PCR reaction (intra-experiment) is generally accepted as being reproducible (Yuen et al., 2002) (Stahlberg et al., 2003), however, the reproducibility of multiple Q-PCR reactions (inter-experiment) has been a significant problem (Freeman et al., 1999). Many of the issues associated with the reproducibility of Q-PCR reactions are related to the RT conversion of RNA to cDNA. This process is poorly understood (Stahlberg et al., 2004a) and considered the step most likely to produce variation in the final Q-PCR result (Stahlberg et al., 2004a) (Bustin and Nolan, 2004a). There is no one accepted method for the RT step and much of the variation in results can be attributed to the type of primers used (Zhang and Byrne, 1999) (Stahlberg et al., 2004a), the enzyme utilised (Stahlberg et al., 2004a) (Stahlberg et al., 2004b) and the potential for mRNA to form secondary structures which may cause inefficient priming during the reaction (Stahlberg et al., 2004a).

There are 3 main type of primers used in RT reactions; random hexamer primers, oligo dT primers and primers specific for the gene of interest. Random hexamer primers are 6 bases long, contain all possible base pair sequences (Kubista et al., 2006) and will copy all RNA (Kubista et al., 2006). The random primers can bind to more than one point along the transcript, yielding more than one cDNA transcript per original target (Bustin et al., 2005). However, the majority of the cDNA will be derived from ribosomal RNA (rRNA), since rRNA makes up approximately 90% of total RNA extracted (Bustin et al., 2005) (Kubista et al., 2006). If the mRNA sequence of interest is in low abundance, random priming would not be the optimal choice as the transcription of the rRNA may compete with the transcription of the mRNA of interest (Bustin et al., 2005). Zhang et al (Zhang and Byrne, 1999) demonstrated that there was a 19-fold overestimation of transcript levels when using random hexamer primers compared to sequence specific primers, and this method has been reported in some studies as the least reliable method for cDNA synthesis (Lekanne Deprez et al., 2002).

Target specific primers are primers that are designed to specifically synthesise the cDNA sequences of interest (Bustin and Nolan, 2004a) and provide the most sensitive measure for quantitation (Lekanne Deprez et al., 2002). This specificity, however, means that a separate reaction for each target of interest is required (Bustin and Nolan, 2004a) (Bustin and Mueller, 2006) (Kubista et al., 2006). Consequently, normalisation to housekeeping genes is not possible with this approach as multiple reactions would be required to amplify each gene.

Oligo dT primers hybridize to the poly(A) tail present on most eukaryotic mRNAs (Kubista et al., 2006) and is, therefore, more specific than random hexamer priming (Bustin and Nolan, 2004a), but Oligo dT priming requires high quality RNA with little degradation (Bustin and Nolan, 2004a) (Kubista et al., 2006). The priming may be inefficient if any secondary structures are present in the mRNA (Bustin and Nolan, 2004a), however, the poly(A) tail target sequences does not easily fold into higher order structures (Kubista et al., 2006). Consequently, the priming of oligo dT is considered to be more efficient and less dependent on temperature compared to specific or random primers (Kubista et al., 2006). Oligo dT has, therefore, been reported as the most reliable method to obtain an accurate cDNA representation of the mRNA pool (Bustin and Mueller, 2006). Since the RNA used in this experiment was to be immediately extracted from cell lines with limited degradation and the quality of the RNA could be checked using gel electrophoresis and accurately quantified using the NanoDrop. Oligo dT priming was consequently chosen as the primer to be used in the RT reactions presented in this study.

Both Bustin and Nolan 2004 (Bustin and Nolan, 2004a) and Wong and Medrano 2005 (Wong and Medrano, 2005) suggest that the minimum criteria for accurate quantitation of gene expression, especially for genes of low expression, is 2 separate experiments run in triplicate. As the RT step is the highest source of error (Stahlberg et al., 2004a) (Bustin and Nolan, 2004a), Stahlberg et al 2004 (Stahlberg et al., 2004a) further concludes that substantially higher experimental accuracy is achieved when replicates are performed on samples from different RT steps instead of replicates performed from the same RT reaction. The results described in this chapter are the combination of 3 independent RNA extractions, subsequent cDNA syntheses and Q-PCR reactions completed in triplicate, thus exceeding the minimum criteria and confirming the results obtained.

Another issue that must be considered prior to conducting Q-PCR experiments, is whether non-specific or specific probe based assays are to be utilised. Non-specific detection uses dyes, such as Sybr Green®, which bind to double stranded DNA after the elongation step of the PCR reaction and emit a fluorescent signal (Ishiguro et al., 1995). Non-specific amplification is usually cheaper and can be incorporated into existing PCR protocols by simply adding the fluorescent dye. However, as this is a non-specific measure, any double stranded DNA, including primer dimers, will also give off a recordable fluorescent signal, affecting the final results (Ishiguro et al., 1995). This issue can be overcome by melt curve analysis following the completion of the PCR

reaction (Ginzinger, 2002) (Ririe et al., 1997) (Kubista et al., 2006), but will not remove the fluorescence in no template controls caused by any non-specific binding (Bustin and Nolan, 2004a). If the non-specific binding is substantial, it could lead to a positive result in the negative control, and nullify the experiment.

Specific detection in Q-PCR requires an antisense fluorescent probe to be synthesised for each gene of interest. The probe exists in a quenched state, giving off no fluorescence until the polymerase reaches the section of DNA where the probe is bound. Once the polymerase reaches the probe it releases the quencher molecule, allowing the fluorescence to be emitted and measured (Kubista et al., 2006). This removes the need for sequencing following the PCR (Bustin and Nolan, 2004a). Additionally, multiple genes can be assayed in the same reaction with the use of different fluorophores (Bustin et al., 2005; Bustin and Nolan, 2004a) (Ginzinger, 2002). Any non-specific amplification, such as primer dimers or contaminations, will not affect the fluorescent recording in this type of reaction, unless they occur in a sufficient proportion to alter the reaction efficiency (Bustin and Nolan, 2004a). This, however, can be overcome by stringent testing of RNA for any DNA contamination, which will reduce the risk of non-specific amplification. Amplification products from Q-PCR experiments can also be electrophoresed on an agarose gel to ensure no non-specific bands, including primer dimers, appear (Kubista et al., 2006).

The fundamental measure used to quantify relative Q-PCR is the cycling threshold (Ct) value. Ct is the point at which fluorescence can be detected above a threshold, set to be higher than the background. Previously, Ct has been the most commonly reported parameter when publishing Q-PCR results (Bustin and Nolan, 2004a). However, the threshold at which the Ct value is obtained is usually chosen by the user and as the background fluorescence can be altered by changing reaction conditions, reporting Ct value alone can lead to results that are not consistently reproducible across a number of separate experiments (Bustin and Nolan, 2004a). This problem has been eliminated by current Q-PCR thermocyclers with a function to automatically correct for background (Ginzinger, 2002) (Bustin and Nolan, 2004a), but if the data is to be used quantitatively, it is still considered essential to apply a normalisation to the data to account for differences between samples, such as variations in starting material and reaction efficiencies (Bustin et al., 2005).

Normalising Q-PCR data remains one of the most difficult problems associated with the technique (Dheda et al., 2004), and determining the most suitable type of analysis for a

specific set of experiments requires careful consideration (Bustin and Mueller, 2006). The two major types of data analysis routinely used are 'relative or comparative quantitation' or 'absolute quantitation' (Rubie et al., 2005). Relative quantitation measures changes in a gene of interest relative to a reference gene known as a calibrator (Wong and Medrano, 2005), which is often a housekeeping gene. The value is expressed as a change in Ct value between the gene of interest and the calibrator and there are a number of published methods for measuring this change, such as ΔCt or $\Delta\Delta$Ct. However, measuring a change relative to a housekeeping gene can lead to discrepancies in the results created from fluctuations in the housekeeping gene expression across treatment groups. This will be discussed in detail below.

Recently, the idea of a constitutively expressed housekeeping gene, particularly across many different treatment groups or patient samples, has been challenged and demonstrated in Q-PCR experiments (Bustin, 2000) (Gibbs et al., 2003) (Rubie et al., 2005) (Valenti et al., 2006) (Mogal and Abdulkadir, 2006). GAPDH is a gene that has been predominantly used as a constitutive housekeeping gene, however, Valenti et al (Valenti et al., 2006) recently published work demonstrating GAPDH expression is altered in different cancer states and upon chemotherapeutic drug administration. Furthermore, using Q-PCR, this group demonstrated that the administration of amino bisphosphonates, bone reabsorption drugs showing anticancer properties, to breast and prostate cancer cell lines significantly increased the expression of GAPDH in a dose dependent manner. In this case, Valenti et al suggest that GAPDH could be used as a marker for observing the effects of bisphosphonates on cancer cells, therefore abolishing its use as a constitutively expressed housekeeping gene in the experiment conducted by Valenti et al (Valenti et al., 2006).

Mogal and Abdulkadir (Mogal and Abdulkadir, 2006) used Q-PCR to examine the effect of the histone deacetylase inhibitor Trichostatin-A (TSA) on the expression of a range of housekeeping genes. This is particularly relevant as BuA has also been demonstrated to be a histone deacetylase inhibitor, and inducer of differentiation in human CRC cell lines (Tsao et al., 1983) . This group demonstrated a significant change in expression of the housekeeping genes β-Actin, GAPDH and 18S rRNA in both rat and human prostate cancer cell lines and in human breast cancer cell lines.

Similarly, Rubie et al (Rubie et al., 2005) tested a variety of housekeeping genes for changes in expression of normal colon and colorectal cancer samples representing different stages of CRC development using Q-PCR. They demonstrated that both 18S

and GAPDH had 1.5 fold increased expression in the cancer samples compared to normal colon tissue, whereas β-Actin remained unchanged. However, this ratio changed for each of the housekeeping genes when they investigated different tumour types, such as stomach cancer and liver metastases. Together these studies demonstrate the need to validate housekeeping genes in every experiment or treatment group.

It is also imperative when using relative quantification that the reaction efficiencies of the housekeeping gene and the targets are equal (Bustin and Nolan, 2004a) and preferably above 90% (Ginzinger, 2002). Testing of the amplification efficiencies can be conducted by performing a dilution of template, conducting a Q-PCR, and plotting Ct as a function of log[10] template concentration/dilution. Reaction efficiency is calculated using the equation: (Ginzinger, 2002) (Wong and Medrano, 2005) (Kubista et al., 2006) (Yin et al., 2001)

$$\text{If: Exponential amplification} = 10^{-(1/\text{slope})}$$

$$\text{Efficiency} = [10^{-(1/\text{slope})}]-1$$

Despite the outlined drawbacks, relative quantitation can be used very successfully if appropriate validation assays on the housekeeping genes have been conducted. These validation assays are required for every disease state or treatment type being investigated (Dheda et al., 2004). It is also possible to compare a panel of housekeeping genes, which has been shown to increase the experimental accuracy (Bustin and Nolan, 2004a) (Bustin and Mueller, 2006). This level of validation is particularly worthwhile when investigating a suite of genes in a small number of treatments or conditions.

The other major method for the quantitation of Q-PCR experiments is absolute quantitation, which employs the use of a standard curve. Absolute quantitation does not rely on or require the use of housekeeping genes (Ginzinger, 2002). In this type of analysis a standard curve is created from known amounts of cDNA with the same sequence as the unknown samples. The cDNA usually arising from a RT-PCR reaction or extraction from plasmids, is quantified and represented as “copy numbers”. The standard curve is created by performing 10-fold serial dilutions (Bustin, 2000) usually in the range of 10^{10}-10^{1} copies (Wong and Medrano, 2005). Values of the unknown samples can be determined from this standard curve to give copy numbers per cell, total RNA, or unit of mass of tissue (Bustin, 2000). High reaction efficiencies are crucial to absolute quantitation (Ginzinger, 2002) and can be calculated from the slope of the

standard curve using the equation; Efficiency = $[10^{-(1/slope)}]$-1, as mentioned earlier in this Chapter. A draw back of this approach is that a standard curve must be produced each reaction, however, this can be beneficial as samples from different Q-PCR reactions can be compared easily as the results for each sample are based on a constant (Wong and Medrano, 2005), which is not easily achieved using the relative quantitation method.

Both relative and absolute quantitation methods have strengths and weaknesses, and the best type of analysis depends largely on the experiment. Since this investigation focussed on a single gene of interest in a number of different treatment groups, specific probes were used in Q-PCR experiments and analysed using absolute quantitation. As mentioned earlier in this Chapter, it is not considered necessary to measure housekeeping genes in this type of analysis. (Ginzinger, 2002), however, suggest that the inclusion of a 'loading control' to ensure no pipetting errors have occurred with any step, including RNA quantitation, RT or Q-PCR, is important for accurate Q-PCR results and that a control gene would identify these errors. Consequently, to show that all RNA samples were RT transcribed equally the expression of GAPDH and 18S, expression levels were also examined, but not used for quantitation.

5.3 Methods for optimisation and verification of Q-PCR

5.3.1 PCR of gDNA for Creation of the Standard Curve

The following procedures were conducted to produce the standard curve which was used in all Q-PCR reactions. gDNA obtained in Chapter 3 was used in an PCR increased by 4 fold to produce a large volume of amplicons.

Each 100μl PCR reaction consisted of: 2μl of dNTP (10mM), 10μl NH_4 buffer (Bioline, Aust.), 1.5mM $MgCl_2$ (Bioline, Aust.), 4μl of forward primer (0.1μg/μl), 4μl of reverse primer (0.1μg/μl), 0.8μl (1 unit) of Taq polymerase (Bioline, Aust.) with the total volume made up to 96μl using molecular grade water. A master mix of all reagents listed above was generated immediately prior to the PCR reaction to reduce errors associated with pipetting small volumes. Ninety-six microlitres of the master mix was added to 4μl of HT29 gDNA obtained from Chapter 3.

Cycling parameters were optimised for each primer set using a Hybaid PCR Express thermocycler.

The GPCR43 protocol consisted of:

94°C for 3 minutes

94°C for 30 seconds
55°C for 30 seconds
72°C for 60 seconds
— 40 cycles

72°C for 5 minutes
10°C for 10 minutes

Purification and quantitation of gDNA was conducted as per 2.3.6 and 2.3.7.

5.3.2 Calculation of PCR copy number to construct a standard curve

Using the concentration calculated in 2.3.8 and the biopolymer calculator software found at http://www.basic.northwestern.edu/biotools/oligocalc.html, the molecular weight of DNA was calculated by providing the program with the DNA sequence and absorbance at 260nm. Using the formula below, copies/ml were calculated and samples diluted to contain 10^7copies/μl. These were serially diluted to 10^1 copies/μl to construct the standard curve (Ginzinger, 2002) (Kubista et al., 2006)

$$\text{Copies/ml} = \frac{6.023 \times 10^{23} \times [C] \times OD_{260}}{\text{Molecular Weight}}$$

Once the Q-PCR reaction was completed, the ABI Sequence Detection software v1.3 for Applied Biosystems Real Time PCR System 7300 (ABI Software) constructed a line of best fit for the standard curve and reported the slope and r^2 value.

5.3.3 Gel electrophoresis of standard curve

An aliquot (5μl) of Q-PCR reaction mix was added to 1μl of DNA loading buffer. The diluted samples were loaded onto an agarose gel (1.5% (w/v) in 1X TAE) then electrophoresed at 100V for approximately 30-40 minutes. A New England Bioscience 100bp ladder was used as a size indicator. Gels were then stained in 1μg/ml Ethidium Bromide for 10 minutes and destained in DEPC-MQ H_2O for 3 minutes. Gels were then photographed using a Kodak DC265 digital camera under U.V. light to visualise the cDNA bands.

5.3.4 Dilutions of starting RNA template

HT29 RNA was diluted in molecular grade water to yield concentrations of 1ng/μl 10ng/μl, 100ng/μl, 1μg/μl and 5μg/μl. 1μl of each concentration of RNA was converted to cDNA according to 2.2.2-2.3.1 and used in a Q-PCR reaction as per 2.4.1.

5.3.5 Calculation reaction efficiencies for the standard curve and the test samples

The starting template used for the standard curve was generated from a PCR amplification and the product was subsequently purified for use in the Q-PCR experiment. The samples tested were cDNA generated from RT reactions. It was, therefore, important to ensure the reaction efficiencies of the standard curve and the unknown samples were the same, and consequently comparable. An aliquot (1μl) of HT29 cDNA sample was diluted in molecular grade water (Invitrogen, Aust.) in the ratios of 1:5, 1:8 and 1:10. 1μl of each of the diluted cDNA samples and 1μl of neat cDNA was added to a Q-PCR reaction as previously described in 2.4.1. The resulting Ct values were plotted against the log concentrations and the line of best fit applied. The reaction efficiency was calculated using the formula:

$$\text{If: Exponential amplification} = 10^{-(1/\text{slope})}$$

$$\text{Efficiency} = [10^{-(1/\text{slope})}]-1$$

5.3.6 Quantitation of GPCR43 on 6 CRC cell line extracts

Dilutions (1:10) of cDNA were made and 1μl of the diluted template was added to the Q-PCR experiment as described in 2.4.1. The values for each of the 6 CRC cell lines were obtained by calculating initial copy number using the standard curve. As discussed in the methods section, initial values were multiplied by 200 for any cDNA diluted 1:10 to give copies/μg total RNA. Once the results had been collected it was clear that some of the values from the 6 CRC cell line extracts were lower than the smallest dilution of the standard curve (10^1). Consequently the experiment was repeated with undiluted cDNA to increase the raw values obtained. In experiments with undiluted cDNA added as template, the initial values were multiplied by 20 to give copies/μg total RNA using the same methodology described previously.

The results presented are the combination of 3 independent RNA extractions, with subsequent cDNA syntheses and Q-PCR reactions completed in triplicate.

5.3.7 Measurement of housekeeping gene expression

1:10 dilutions of cDNA were made and 1μl of the diluted template was added to the Q-PCR experiment as described in 2.4.1. The Ct values for each of the 6 CRC cell lines were presented.

5.3.8 Statistical analysis

All analyses were conducted using One-Way ANOVAs were performed with Tukey's post hoc testing and significance is indicated by values $p<0.05$ unless otherwise stated. All statistical analyses were conducted using GraphPad Prism 5 software for Windows.

5.4 Results

5.4.1 Optimisation and verification experiments

Initially, a standard curve was run to ensure the success of the Q-PCR reaction and to measure the r^2, slope and reaction efficiency of the standard curve. Figure 5.1 shows the initial Ct values from a standard curve and Figure 5.2 shows the standard curve derived from this experiment. An r^2 value 0.997 was calculated, indicating reproducibility within the triplicates and accurate dilutions of the starting cDNA from 10^7-10^1copies/μl. The slope of this curve was -3.4 indicating a reaction efficiency of 94.5%. The amplification products generated from the standard curve were also visualised on an agarose gel. The Figure 5.3a shows each dilution of the standard curve gives an amplicon of approximately 120bp, with no other amplicons detected. The products were quantified using Scion Image software (Figure 5.3b).

The 10^7-10^1 dilutions of the standard curve were used as a template for the RT-PCR using the primers FWD3 and REV1, which are nested within the full length sequence as seen in Figure 5.4. Figure 5.5a shows strong amplification of the 330bp GPCR43 amplicon with a decrease in the intensity of the bands as the dilutions of the starting template decreased from 10^7-10^1. The quantitative results from densitometry analysis using Scion Image are shown in Figure 5.5b.

To calculate the reaction efficiency of the unknown samples, dilutions of HT29 cDNA were used in a Q-PCR, the resulting Ct values were plotted against the log dilutions, and the line of best fit applied. The slope of this line was -3.4 and the reaction efficiency was calculated to be 94.5% (Figure 5.6), which is equal to the reaction efficiency of the standard curve.

Before accurate quantification was possible, it was vital to ensure that no contaminating gDNA was present in the cDNA samples which could lead to false positive results and inaccurate quantitation. Non transcribed cDNA from each of the 6 CRC cell lines was added to a Q-PCR reaction. No amplification occurred with the vast majority of the non transcribed cDNA, but in this experiment a small number of non-transcribed cDNA replicates recorded very high Ct values after 38 cycles. As this amplification did not occur in all replicates of any one triplicate or in the same cell line across any of the 3 independent experiments, the RNA generated for all 6 CRC cell lines was considered free of gDNA contamination. This conclusion will be explained in more detail in the discussion. No template controls were also negative for amplification.

RNA of differing concentrations; 1ng/μl 10ng/μl, 100ng/μl, 1μg/μl and 5μg/μl was converted to cDNA and used in Q-PCR reactions. Results, shown in Figure 5.7, show the initial 'copies of GPCR43' obtained from the standard curve prior to any correction for dilutions or starting template. Samples of 1ng/μl, 10ng/μl and 100ng/μl all produced less than 10 copies of GPCR43, which is less than the minimum number of copies that can be quantified with confidence as 10^1 is the lowest value on the standard curve. Samples of 1μg/μl and 5μg/μl of RNA gave values of 19 ± 4 and 18 ± 2 respectively. The results from this section determined which starting concentration of RNA was most efficient and this concentration was used in future experiments.

5.4.2 Quantitation of GPCR43 expression on 6 CRC cell lines

Results of the quantitation of GPCR43 from the cDNA from the 6 CRC cell lines is shown in Figure 5.8 for template diluted 1:10 and Figure 5.9 for undiluted cDNA. Results for 1:10 diluted cDNA show that the highest level of expression was observed in the HT29 cells with 8322 ± 1202 copies/μg total RNA, followed by T84 cells with 203 ± 36 copies/μg total RNA, CaCo2 cells with 80 ± 26 copies/μg total RNA and LIM1215 cells with 38 ± 8 copies/μg total RNA. SW480 and HCT116 cells produced no detectable level with amplification. With undiluted cDNA, highly similar results were observed in each cell line as seen in Figure 5.9. The no template controls were negative upon amplification, indicating that contamination did not affect these results.

The expression of the 2 housekeeping genes; 18S and GAPDH was assessed using Q-PCR from cDNA synthesised from 3 independent RNA extractions (Figure 5.10). The cDNA from each of the cell lines amplified at approximately 15-20 cycles (Figure 5.10)

demonstrating the integrity of the cDNA. The results for each housekeeping gene were calculated to not be significantly different in any run or cell line using One-Way ANOVA performed with Tukey's post hoc testing.

To determine which of the housekeeping genes tested had the least variation the average Ct value from all samples was calculated to give *mean Ct* and the percentage variation in each cell line was then calculated by measuring precision defined as SD/*mean Ct* value x 100. The percentage CV for 18S and GAPDH was 4.2% and 7.2% respectively.

5.5 Discussion

5.5.1 Optimisation and verification experiments

5.5.1.1 Validation of the standard curve

The results obtained in this Chapter demonstrate that the Q-PCR standard curve reaction is occurring in a highly efficient manner, as indicated by a reaction efficiency greater than 90% (Kubista et al., 2006). Despite this, non-specific amplification can occur in Q-PCR reactions and has the potential to affect the reaction efficiency (Bustin and Nolan, 2004a). To determine if this was a confounding factor in the current experiment, amplicons generated from the Q-PCR reaction of the standard curve were visualised using agarose gel electrophoresis (Kubista et al., 2006). The amplicons in Figure 5.3a were all approximately 120 base pairs in length, demonstrating consistent amplification in all dilutions of the standard. The absence of any additional bands on the agarose gel demonstrates that non-specific amplification is not occurring.

When the amplicons produced from the Q-PCR reaction of the standard curve were run on an agarose gel, there were observable decreases in band intensity with decreasing amounts of starting template (Figure 5.3a). These results were quantified using the densitometry software Scion Image and are shown in Figure 5.3b. Similarly, a decrease in the intensity of bands with decreasing starting template was observed when the amplicons produced by the RT-PCR reaction of the standard curve using nested primers (Figure 5.4) were electrophoresed (Figure 5.5a) and quantified (Figure 5.5b). This indicates that less amplification product was produced from less starting template and further demonstrates the integrity of the standard curve.

Although the standard curve was constructed to absolutely quantify copy number of GPCR43, it can also serve as a positive control. Each decrease in starting template (10^7-10^1) should change the C_t value by approximately 3.3 cycles (Larsen et al., 2002), which can be seen in Figure 5.1. Furthermore, the greatest dilution of the standard curve (10^1) should be the lowest value quantified with confidence (Bustin and Nolan, 2004a). Collectively, all of the tests conducted in this section demonstrate the accuracy and integrity of the standard curve, and will enable the reliable and reproducible analysis of GPCR43 expression in a number of samples and treatments.

5.5.1.2 The reaction efficiency of the standard curve and unknown samples are comparable

Unknown samples may contain inhibitors which are not present in purified standards, therefore it is important to test the efficiency of both the standards and the unknown samples (Stahlberg et al., 2003). This can be achieved by running dilutions of the unknown samples to calculate the efficiency of that reaction to ensure the standard curve and the unknown samples are comparable (Stahlberg et al., 2003). A similar approach was used by Livak and Schmittgen (Livak and Schmittgen, 2001) who used cDNA dilutions to show the reaction efficiencies of 2 genes were the same. In the current experiment the reaction efficiency of the unknown samples was calculated to be 94.5% (Figure 5.6). This value was above 90% and comparable to the reaction efficiency of the standard curve, shown in Figure 5.1, the unknown cDNA samples could be confidently quantified using the standard curve (Ginzinger, 2002).

5.5.1.3 No template controls are free of contamination

In Chapter 4, it was stated that PCR experiments should only be conducted to a maximum of 40 cycles to avoid non specific amplification. However, in the current study Q-PCR reactions were run for 45 cycles and RT-PCR reactions were run to 40 cycles. Bustin and Nolan 2004 (Bustin and Nolan, 2004a) state that if the Ct value of any unknown sample lies within the region of 37-39 cycles the reaction should be run to 45 cycles to ensure the NTC is negative. Many of the samples in this study were amplified very late in the Q-PCR and had Ct values greater than 35. Therefore, to ensure that the detection observed at high Ct values were genuine, and not due to non specific amplification, the Q-PCR was run for 45 cycles. This approach also enabled the observation of the entire profile of each reaction which would not have been possible for the samples with high Ct values if the reaction had been stopped at 40 cycles. Samples

with values greater than 40 cycles were not accepted for any analysis, due to the risk that these may be due to non-specific amplification, such as primer dimers.

5.5.1.4 RNA is gDNA free

No template control samples or other negative controls such as non-transcribed cDNA can sometimes record non specific detectable fluorescence when approaching the high Ct values at end point of a Q-PCR reaction. In this experiment a very small number of non-transcribed cDNA replicates demonstrated amplification, but these were very high Ct values which were all greater than 38 cycles. Bustin and Nolan (Bustin and Nolan, 2004a) state that "the whole question of how to interpret a positive NTC is the subject of many a heated debate" and that "interestingly no guidelines have been published on this matter". They also conclude that if a NTC has a very high Ct value, which is substantially different from the Ct value of the unknown, that is greater than 5 cycles, it is legitimate to ignore the very high Ct from the NTC. As the amplification that occurred in the non-transcribed cDNA did not occur in all replicates of any individual triplicate or in the same cell line across any of the 3 independent experiments the RNA generated for all 6 CRC cell lines was considered free of gDNA contamination.

5.5.1.5 1μg of RNA is required for cDNA synthesis

Increasing quantities of RNA were added to the cDNA synthesis step to determine the optimal amount of RNA for this reaction. The initial results were calculated by the ABI software to give "copies of GPCR43/amount of RNA added". Initially, no corrections were made for the difference in starting concentration, to identify which quantities of RNA were insufficient to generate 10 or more "copies of GPCR43". As 10 copies is the minimum of the standard curve, any values that do not lie on the standard curve cannot be considered accurate (Bustin and Nolan, 2004a). Results from this Chapter demonstrate that the minimum amount of RNA required for synthesis to cDNA for investigating the expression of GPCR43 is 1μg of total RNA, as lower amounts resulted in raw Ct values under 10 copies of GPCR43 as seen in Figure 5.7.

Since every doubling of RNA decreases the Ct value by 1 (Bustin and Nolan, 2004a), it would be expected that there would be a dose dependent increase in copy number with increasing RNA template. The results in Figure 5.7 do not show this dose dependent change, when RNA quantity increased from 1ng to 10ng or from 10ng to 100ng nor 100ng to 1000ng (1μg). Curry et al (Curry et al., 2002) showed that the reaction

efficiency of the RT step is significantly lower when the target templates are rare, which may account for the observation in the current study. The results from the expression of GPCR43 on 6 different CRC cell lines, discussed later in this Chapter, show that GPCR43 is a low abundance transcript, so GPCR43 may be largely undetectable in the current study until 1μg of RNA is added to the RT step. A phenomenon known as the Monte Carlo Effect could also partially account for this observation. The Monte Carlo effect suggests that the lower the abundance of the template the less likely the true abundance will be reflected in the final Q-PCR results (Karrer et al., 1995) (Bustin and Nolan, 2004a). If the template is very low, then a situation of primer excess will occur. Since the efficiency of primer binding is partially dependent on the availability of template, samples with low abundance of template will have modified reaction efficiencies (Karrer et al., 1995). Therefore, despite the increase in template abundance from 1ng to 100ng, no increase in final copies of GPCR43 following Q-PCR was observed.

Both 1μg and 5μg of total RNA produced Ct values that were within the dilutions of the standard curve, however, both 1μg and 5μg generated initial Ct values which were statistically similar (values from 1ng-5μg were compared using One way ANOVA with Tukey's Post Hoc testing); indicating a "saturation" of the cDNA synthesis reaction occurring at 5μg of total RNA. Curry et al (Curry et al., 2002) and Stahlberg et al (Stahlberg et al., 2004b) have demonstrated that the synthesis of cDNA can be negatively affected by non-specific nucleic acid in the reaction, usually resulting from high concentrations of rRNA. This observation could explain why both 1μg and 5μg total RNA yield similar copies of GPCR43, despite different starting total RNA amounts. Consequently, 1μg/μl of total RNA was chosen as the concentration of RNA that would produce the most accurate results.

5.5.2 Quantification of GPCR43 expression on 6 CRC cell lines

5.5.2.1 RNA was equally transcribed to cDNA in all samples

As outlined in the Introduction of this Chapter, the RT of RNA to cDNA is considered the step most likely to lead to errors in the final Q-PCR results (Bustin and Nolan, 2004a) (Ginzinger, 2002). To overcome this issue the measurement of a control or housekeeping gene is required to ensure the uniform transcription of RNA to cDNA (Ginzinger, 2002). However, this requires the assumption that housekeeping genes are constitutively expressed. It has been well documented, and discussed in detail in the

Introduction of this Chapter that housekeeping gene expression may not be consistent across all samples and treatment types, and makes relative quantification difficult to analyse. To avoid this assumption, absolute quantification has been utilised, however, housekeeping genes were still employed to show even cDNA synthesis across all samples. This does rely on the assumption that the housekeeping genes are constitutively expressed. Nonetheless, investigating housekeeping gene expression as an additional step to absolute quantitation was conducted to improve the reliability of the results from the Q-PCR experimentation at or above the currently accepted convention.

The data in this section was presented as Ct values, and although there are limitations in presenting Ct values alone, as outlined in the Introduction of this Chapter, it is usually considered necessary to apply a normalisation to the data to account for differences in starting material, reaction efficiencies, and differences between samples (Bustin et al., 2005). However, in these experiments, the Ct values have been corrected for background by ABI software, eliminating user error (Bustin and Nolan, 2004a), and the starting RNA template was carefully quantified. As the data was not used for quantification it was not essential for the reaction efficiency to match the target gene of interest. The Ct value is directly proportional to the amount of starting RNA template (Ginzinger, 2002) and, therefore, when used in a non-quantitative manner, such as a 'loading control' (Ginzinger, 2002) (Pfaffl, 2001) for RNA starting concentration and uniform cDNA synthesis, Ct values were deemed adequate. In can be clearly observed in Figure 5.10 that uniform synthesis of cDNA from RNA occurred, as shown by stable expression levels of both 18S and GAPDH housekeeping gene expression.

5.5.2.2 18S was the most stable housekeeping gene in the current study

β-Actin was used in the previous Chapter, and although expression was stable, there were small variations in the expression of this gene using quantitative analysis of the gel image. Consequently, the expression of two other routinely used housekeeping genes were tested; GAPDH and 18S. The limitations of housekeeping genes have already been outlined in the Introduction of this Chapter. It is, however, thought that rRNA molecules are more reliable housekeeping genes compared to mRNA targets (Goidin et al., 2001) (Schmittgen and Zakrajsek, 2000) (Bhatia et al., 1994). 18S and 28S expression levels are thought to remain more constant than mRNA across different conditions due to the high abundance of rRNA (Bustin, 2000). In the current study, both housekeeping genes showed some variation, but most of this variation was under 10% (CV value as described in section 5.4.2). 18S had less than 4.2% variation across all

samples and experiments and although GAPDH had more variation, this variation was still low, with a CV value of 7.2%. This result indicates that despite both housekeeping genes showing even expression in all samples, 18S was the most stable and consequently was used for future experimentation.

18S is generally not considered to be an appropriate housekeeping gene for experiments using oligo dT primers as 18S is rRNA and does not posses a poly(A) tail to bind to the primers (Ginzinger, 2002) (Bustin and Nolan, 2004a) (Bustin and Mueller, 2006). However, Stahlberg et al (Stahlberg et al., 2004a) demonstrated that in the absence of any primer in the RT reaction, cDNA synthesis still occurred, although less efficiently. This research group concluded that RNA, or potentially dNTP's within the RT reaction, are able to act as primers, even if the priming event has a low degree of specificity. Frech and Peterhans (Frech and Peterhans, 1994) have also reported the transcription of cDNA in the absence of any primer in the RT reaction. This group concluded that total cellular RNA extractions contain sufficient small RNA fragments to synthesise a diverse range of cDNAs, irrespective of the reverse transcription enzyme or RNA extraction method used. Aside from the small RNA fragments acting as primers for the reaction, it is likely that the oligo dT primer is able to partially bind to poly(A) stretches in the sequence to allow transcription. The results from the current study have demonstrated that the RT of 18S can occur with oligo dT primers in the RT reaction. Furthermore, the RT of 18S in this experiment is consistent and occurs in uniform manner across all samples, and does so in a more stable manner than GAPDH. This is why 18S was chosen as the housekeeping gene in future experiments in the current study.

5.5.2.3 Experimental summary

As indicated, the most important aspect of Q-PCR is to conduct well controlled, carefully planned and executed experiments (Bustin and Nolan, 2004a) that remain consistent across all samples. The controls described in this Chapter produced reliable and reproducible results and, therefore, the basis for further experimentation using Q-PCR to investigate GPCR43 expression in a variety of samples and treatments was established.

5.5.2.4 The expression of GPCR43 can be detected in HT29 cells but is expressed in very low levels in all other cell lines tested

HT29, CaCo2, LIM1215 and T84 cells were demonstrated to show expression of GPCR43 in the experiments described in the current Chapter. The results from the RT-PCR experiments from Chapter 4 and Q-PCR results from this Chapter produced broadly consistent results, with the only discrepancy occurring in the LIM1215 cell lines. In LIM1215 cells, the RT-PCR did not detect any expression of GPCR43, whereas the Q-PCR did. This is most likely due to the increased sensitivity of Q-PCR compared to conventional RT-PCR. As previously described in this Chapter, the lowest dilution of standard curve is the smallest value that can be quantified with confidence. The initial values for every cell line, except HT29, were below 10^1 copies, which indicates that the expression detected in CaCo2, T84 and LIM1215 cells are below the level of detection for accurate quantitation. More template was added to the Q-PCR in an attempt to increase the initial values above the minimum 10 copies, however despite increases in copy numbers, only the T84 initial values were increased above the minimum level. These results indicate that all cell lines tested, aside from HT29 cells, have very low expression levels of GPCR43. It was interesting to note that all cell lines possess a copy of the GPCR43 gene (results from previous Chapter) although the expression profile across each cell line varies remarkably. In the 6 CRC cell lines tested there does not appear to be a correlation between GPCR43 expression and disease progression, it would be of great interest to expand this study to include more cell lines to elucidate if there are any commonalities between cell lines and their expression of GPCR43. Furthermore, it would be of great interest to investigate the receptor expression on normal and cancerous human patient samples to determine if any changes in expression occur with the progression of malignancy. This will be addressed in Chapter 9.

5.5.2.5 Other published results relating to GPCR43

It is difficult to directly compare the results of different PCR publications, even if similar methodology has been used. Bolufer et al (Bolufer et al., 2001) conducted a multi-centre study, with 18 laboratories detecting the levels of PML-RARα using 1 of 4 methods of Q-PCR. This study showed that identical samples assayed using exactly the same methodology in different laboratories yielded different detection levels of this gene. The authors suggest that some of the variation may be due to sample degradation, but may also be due to differences in the handling and procedure employed by each laboratory even though the methodology was consistent. Despite the

difficulties with direct comparisons, the current study has attempted to relate the results obtained in this work with other published work relating to GPCR43.

Although Hong et al (Hong et al., 2005) used semi-quantitative PCR to analyse the expression of GPCR43, they conducted experiments to determine the exponential phase of the reaction. For GPCR43, they reported cycle 35 of the RT-PCR to be in the exponential phase of the reaction. In the current study, the Ct values for each of the 6 CRC cell lines ranged from 30-38. These values show that the exponential phase of the Q-PCR conducted in this Chapter was comparable to that observed by Hong et al (Hong et al., 2005).

In 2003, 3 experimental papers were published reporting the expression and functional characterisation of GPCR43 (Le Poul et al., 2003) (Brown et al., 2003) and (Nilsson et al., 2003). Each of these groups tested the expression of GPCR43, and Le Poul et al (Le Poul et al., 2003) and Brown et al (Brown et al., 2003) also investigated the expression of GPCR41 in a variety of tissue types. They found the expression of GPCR43 to be the highest in immune type cells such as monocytes, neutrophils and PBMCs and GPCR41 to be highly expressed in adipose tissue. Expression of GPCR43 was not investigated in the colon until 3 years later, when Hong et al (Hong et al., 2005) first published expression of GPCR43 in this tissue. Unlike Le Poul (Le Poul et al., 2003) and Brown et al (Brown et al., 2003), Hong et al (Hong et al., 2005) demonstrated that the highest expression of GPCR43 was in adipose tissue and that GPCR41 was not present in these samples. The authors noted this result was different to the published data of Le Poul et al (Le Poul et al., 2003) and Brown et al (Brown et al., 2003) but stated that they "do not know the reason for this discrepancy". Le Poul et al (Le Poul et al., 2003) and Nilsson et al (Nilsson et al., 2003) both used semi-quantitative PCR to investigate gene expression, whereas Brown et al (Brown et al., 2003) used Q-PCR. In their original paper this group represented the expression of GPCR41 using absolute quantitation as copies/ng poly(A) RNA and GPCR43 as arbitrary units. However in the review published by the same first author in 2005 (Brown et al., 2005) the expression of both GPCR41 and GPCR43 was expressed using absolute quantitation as copies/ng poly(A) RNA. The highest expression of GPCR43 occurred in spleen and PBMC cells at approximately 2500 and 2000 copies/ng poly(A) RNA respectively, which can be compared to the highest value of 8322 ± 1202 copies/μg total RNA obtained for HT29 cells produced in this Chapter. Despite both papers using absolute quantitation to represent the data, (Brown et al., 2005) have transcribed only the poly(A) RNA compared to the current study which used total RNA. Poly(A) RNA represents only the

mRNA from RNA extractions and excludes the tRNA's and the highly abundant rRNA. This process can enhance the yield, and subsequently the detection of low abundance mRNA transcripts, but often has many associated issues with the extraction process leading to loss of RNA and difficulties in assessing the quality (Bustin and Mueller, 2006) with the absence of rRNA bands.

5.5.2.6 Summary

The results described in this Chapter provide further evidence for the presence of GPCR43 receptors on HT29 CRC cell lines. The failure to observe expression on GPCR43 on all cell lines tested is of interest. It was shown earlier in this study that all the cell lines, apart from T84 cells, responded to BuA by way of apoptosis and decreased proliferation in varying extents. The absence of GPCR43 on cells that respond to BuA-induced apoptosis and proliferation suggests the BuA receptor and the proliferative and apoptotic processes are not tightly coupled. However, more definitive evidence for this relationship comes from knockdown experiments described in Chapter 8.

6 Modulation of GPCR43 expression occurs with changes to cell confluence

6.1 Introduction

With the establishment of a reliable quantitative measure of GPCR43 gene expression, as described in the previous Chapter, it was possible to investigate the level of GPCR43 expression in a number of cell environments and treatment conditions. In the current Chapter the effect of cell culture confluence on expression of GPCR43 was investigated.

A number of groups have demonstrated that the expression of genes can alter with changes in cell confluency. Fiashi et al (Fiaschi et al., 2001) demonstrated that the expression levels of Low Molecular Weight Protein-Tyrosine Phosphatases (PTPs), which have been implicated in fundamental physiological processes such as growth and proliferation, differentiation, and cytoskeletal function, was upregulated in densely confluent cultures. Lai et al (Lai and Pittelkow, 2004) demonstrated a similar modulation of gene expression with changing cell confluence in normal human keratinocytes. One focus of this group was the investigation of the regulation of a novel wound healing factor, secretory leukocyte protease inhibitor (SLPI). In media containing epidermal growth factor (EGF), this group demonstrated a very large (60%) up-regulation of SLPI at 4 days post-confluence. Furthermore, this group showed up-regulation of vascular endothelial growth factor (VEGF), amphiregulin, cytokeratin-1 (K1) and transforming growth factor-α (TGF-α) gene expression across different cell confluences.

Han et al (Han et al., 2006) investigated gene expression in 40%, 90% and over-confluent kidney 293 cells by extracting RNA from cells at each state of confluence, conducting a reverse transcription reaction and hybridising to cDNA arrays. This group (Han et al., 2006) identified 443 genes that were differentially expressed in 90% confluent and over-confluent cells compared with the 40% confluent cells, which were used as a common reference. Using this technique, they identified that regulation of gene expression falls into two major groups. In the first group the genes were upregulated in 90% confluent cells and downregulated in over-confluent cells compared to the 40% confluent culture. In the other group the genes were downregulated in the 90% confluent cells and upregulated in the over-confluent cells compared to the 40% confluent cultures. The results from this study indicated that the expression levels of a substantial number of genes are altered with changing cell confluence. Han et al (Han

et al., 2006) therefore suggest that the cell confluence status could be determined using analysis of gene expression.

From these findings described above it became apparent that it was important to determine if GPCR43 expression remained constant with changes in cell confluency. To do this, CRC cells were plated at equal starting densities and gene expression measured at 24, 48 and 72 hours after seeding which corresponded to 20-30%, 40-50% and 80-90% confluent cultures.

It was also of interest to ascertain whether any changes in GPCR43 expression due to confluence were related to altered physiological function. Several studies have associated increased cell confluence with an increased differentiation status of cells (Lai and Pittelkow, 2004) (Comalada et al., 2006) (Su et al., 2007) (Poumay and Pittelkow, 1995). Comalada et al (Comalada et al., 2006) plated varying densities of normal epithelial cells (FHC) and a colon cancer cell line (HT29), and measured alkaline phosphatase activity as a marker of differentiation at each of the densities. They demonstrated that confluent cells showed higher levels of alkaline phosphatase than sub-confluent cells. Furthermore, this group showed that the confluent cells had the same alkaline phosphatase levels as differentiated cells indicating that the confluent cells had undergone differentiation.

Interestingly, GPCR43 has been implicated in cellular differentiation by two studies (Senga et al., 2003) (Hong et al., 2005). Therefore, it was hypothesised that any changes in GPCR43 expression observed with an increase in cell confluence may correspond with an increase in differentiation, as measured by alkaline phosphatase activity. To determine if any changes observed in GPCR43 expression with increasing confluence were associated with increased differentiation, alkaline phosphatase activity was measured in cells at each level of confluency. Additionally, it has been well established that CRC cell exposure to BuA induces an increase in differentiation (Gibson et al., 1992) (Toscani et al., 1988) (Leder and Leder, 1975) (Kruh, 1982a; Leavitt et al., 1978). Therefore, it was of interest to investigate BuA-induced differentiation in the current study using the alkaline phosphatase assay.

6.2 Approach

1. To investigate the expression of GPCR43 on HT29 and HCT116 cells with increasing cell confluence.

2. To investigate the differentiation status of HT29 cells with increasing cell confluence.
3. To investigate the differentiation status of HT29 cells following exposure to 5mM BuA.

6.3 Methods

6.3.1 Methods for investigating the confluency of CRC cell lines

Photographs of HT-29 and HCT-116 cells were taken 24, 48 and 72 hours after being seeded using the methodology described in 2.5.1. This enabled the viewing of the cell confluence at each time point and any morphological changes that may have occurred over time.

In the paper by Vecsey-Semjen et al (Vecsey-Semjen et al., 2002) a photo image from a light microscope and a description of 9 different CRC cells was presented and have been used as the basis for descriptors in this study. In the study by Vecsey-Semjen et al (Vecsey-Semjen et al., 2002) the cells were divided into 3 categories and described as epithelial-like, piled up and rounded up. Other descriptors of cell morphology based on those presented by Joseph et al (Joseph et al., 2004) and Bonnotte et al (Bonnotte et al., 1998) have been used in the current study .

6.3.2 Cell culture experiments to determine expression of GPCR43 and during changes in confluency

HT29 and HCT116 cells of 85% confluence were treated with trypsin and plated into 3 flasks each with equal seeding density. Cells were incubated at 37°C, 5% CO_2 for 24, 48 or 72 hours to yield 30%, 50% or 85% confluence respectively. At the end of each time point, RNA was extracted and processed to cDNA for Q-PCR analysis as discussed in 2.2.2-2.3.1 and 2.4.1. The results represent Q-PCR reactions run in triplicate in a total of 3 experiments.

6.3.3 Cell culture experiments to determine the differentiation status of HT29 cells during changes in confluency

Cell culture experiments to investigate cell confluence were conducted as described in the previous section 6.3.2. However, instead of extracting RNA at the end of each time point, the cells were lysed for protein analysis as described in the cell lysis section 6.3.5.

6.3.4 Cell culture experiments to investigate the effects of 5mM BuA on HT29 differentiation

T_{75} flasks of approximately 85% confluence were treated with trypsin, plated at equal seeding density (1:4 split) into T_{75} flasks and incubated for 24 hours at 37°C, 5%CO_2. After 24 hours, media was removed and replaced with normal growth media with or without 5mM BuA, and the flasks incubated at 37°C, 5% CO_2 for 48 hours. Cells were then lysed for protein analysis.

6.3.5 Cell lysis for differentiation assay

Cells were treated with trypsin, counted and the cell pellet collected by centrifugation at 450g for 5 minutes. Cells ($5x10^6$) were then lysed in 1ml of M-mammalian cell lysis reagent (Sigma, USA) buffer containing 1μl of Protease Inhibitor Cocktail for mammalian cells (Sigma, USA) on a plate shaker for 15 minutes at 4°C. The lysed cells were then centrifuged at 12,000g for 15 minutes at 4°C to pellet the cellular debris. The supernatant was removed, placed into a 1.5ml tube and stored at -80°C.

6.3.6 Determination of Protein Concentration

Total protein concentrations from each cell lysate were calculated using the Bicinchoninic Acid (BCA) Assay (Sigma Aldrich, USA. # BCA1-1KT) according to the manufacturer's instructions. A brief description of the protocol has been provided with the names of all solutions as they appear in the Sigma Aldrich BCA kit. Briefly, a 1:20 dilution of solution B was added to solution A to prepare the "Standard Working Reagent" (SWR). Aliquots (100μl) of Bovine Serum Albumin (BSA) standards ranging from 0-1mg/ml were prepared in the cell lysis reagent used for the cell lysis procedure. Cell lysates (25μl) and each standard were added to a clear 96-well plate in triplicate followed by 200μl of SWR. The 96-well plate was then covered and incubated at 37°C for 30 minutes. Following the incubation, plates were read on a Wallac Victor 3 plate reader (Perkin Elmer, Aust.) at an absorbance of 560nm. A standard curve was derived using Microsoft Office, Excel 2003 software and the concentration of each sample was determined using the equation of the line calculated from the standard curve. Samples were then diluted to 4μg total protein for use in determining the amount of differentiation induced in a range of conditions using the differentiation assay.

6.3.7 Differentiation Assay

The fluorescent Alkaline Phosphatase Detection Kit, (Sigma, USA # APF-1KT) was used to measure differentiation according to the manufacturer's directions. Aliquots (80μl) of cell lysates containing 4μg total protein from 6.3.6 were heated at 65°C for 15 minutes in 1.5ml tubes, and then cooled on ice for 2 minutes. An aliquot (20μl) of each sample was added to a 96-well white Nunclon Delta microwell plate (Nunc, Denmark) in triplicate. A control group consisting of 20μl of lysis buffer only was also included. A solution consisting of 1:9 dilution buffer:fluorescence buffer was prepared and 180μl added to each well. Another solution containing 1 volume of 4-Methylumbelliferyl phosphate disodium substrate and 3 volumes of deionised water was prepared and 4 μl of the diluted substrate was added to each well using a multi-channel pipette. The plate was then stored in the dark for 20 minutes to process and read on a Wallac Victor 3 plate reader (Perkin Elmer, Aust.) at 355nm excitation and 460nm emission.

6.3.8 Statistical analysis

All analyses were conducted using One-Way ANOVAs were performed with Tukey's post hoc testing and significance is indicated by values $p<0.05$ unless otherwise stated. All statistical analyses were conducted using GraphPad Prism 5 for Windows.

6.4 Results

6.4.1 Changes with cell confluency

Twenty-four hours after seeding, both HT29 cells and HCT116 cells showed a cell confluence of approximately 20-30% as determined by visual inspection. At this time point both the HT29 and HCT116 cells appeared rounded and small (Figure 6.1 and 6.2).

Forty-eight hours after seeding, both cell lines were approximately 40-50% confluent and an increase in cell size and flattening was observed in both cell lines. HT29 cells at this time point had rounded edges and appeared to have an epithelial phenotype. The HCT116 cells also appeared to have an epithelial phenotype, however, these cells had hexagonal or "sharp edges".

Seventy-two hours post seeding, both cell lines reached approximately 80-90% confluence and had fully flattened out across the bottom of the flask. In both cell lines individual cells have a much greater size compared to the cells at both 20-30% and 40-

50% confluency. In a similar way to the cells observed at 40-50% confluence, both cell lines displayed an epithelial phenotype with rounded edges observed for HT29 cells and hexagonal edges seen for HCT116 cells.

6.4.2 Expression of GPCR43 with increasing cell confluence

In HT29 cells, similar expression of copies of GPCR43/μg total RNA were observed at 20-30% expression and 40-50% expression (Figure 6.3a). There was, however, a large, though not significant, increase in GPCR43 expression at 80-90% confluence (Figure 6.3a). In HCT116 cells, no detectable levels of GPCR43 expression were observed (Figure 6.3b), despite uniform expression of 18S being observed in both HCT116 and HT29 cells (Figure 6.4a and 6.4b). Further experimentation was, therefore, not conducted using HCT116 cells.

6.4.3 Differentiation of HT29 cells with increasing cell confluence

The results summarised in Figure 6.5 show there is no increase in alkaline phosphatase activity with an increase in cell confluence, hence indicating that the differentiation status of HT29 cells is not increased with cell confluence.

6.4.4 Differentiation of HT29 cells following BuA exposure

The results in Figure 6.6 demonstrate a strong and significant increase in alkaline phosphatase activity in cells exposed to 5mM BuA compared with 0mM BuA and the no template control.

6.5 Discussion

6.5.1 Changes with cell confluency

The increase in both HT29 and HCT116 cell confluence was observed over the 3 time points examined (Figure 6.1 and 6.2). The results from this study enabled the determination of the percent confluence at 24, 48 and 72 hours post seeding and also allowed the changes in cell morphology with increasing cell confluence to be observed. Both cell lines are small and rounded 24 hours post seeding (20-30% confluence) and begin to flatten out by 48 hours (40-50% confluence). At the 48 hour time point differences in cell morphology become apparent, in particular the shape of HT29 cells are rounded compared with the HCT116 cells that have hexagonal edges. At the 72 hour time point cells are reaching maximum confluence (80-90%) and in both cells lines the cells have flattened out. The observation of the cell morphology at each confluence

clearly demonstrates the differences in cells as they proliferate and the importance of maintaining cells at constant rates of confluence throughout experimentation.

6.5.2 Alterations in GPCR43 expression changes to cell confluence

Cell confluence, as discussed in the introduction of this Chapter, has been shown to affect the expression of a number of genes. In earlier Chapters the expression of GPCR43 was only investigated at approximately 85% cell confluence to ensure consistency across all cell lines. However, it was of interest to know whether the expression of this receptor was altered with increasing confluency. The results from this Chapter demonstrated that the expression of GPCR43 in HT29 cells was mostly constant at 20-30% and 40-50% confluence, with a large but statistically insignificant increase in the expression at 80-90% confluence (Figure 6.3a). Consequently, it was considered important that all cells must be at the same confluence at the time of RNA extraction to ensure consistency across samples.

No expression of GPCR43 was observed in HCT116 cells at any rate of confluence (Figure 6.3b). This result is not unexpected as GPCR43 expression was not detected in HCT116 cells in Chapter 4 or 5, but confirms that this result is not altered by confluency.

6.5.3 The trend for increased GPCR43 expression following increased cell confluence does not correlate with HT29 cell differentiation

As described in the Introduction of this Chapter, Comalada et al (Comalada et al., 2006) demonstrated greater differentiation in confluent cell populations and consequently linked cell confluence to differentiation status. Interestingly, Senga et al (Senga et al., 2003) connected GPCR43 with differentiation. This group showed the expression of leukocyte-specific STAT-induced GPCR (LSSIG), a novel murine orphan GPCR with high homology to human GPCR43, was significantly increased in M1 leukemia cells during the leukemia inhibitory factor (LIF)-induced differentiation to macrophages. Furthermore, this group showed that cytokine- and lipopolysaccharide-induced differentiation of HL-60 and U937 leukemia cell line to monocytes also increased the expression of GPCR43.

Two years after Senga et al (Senga et al., 2003) implicated GPCR43 in monocyte differentiation, and Hong et al (Hong et al., 2005) conclusively demonstrated using RNAi knockdown techniques that the acetate- and propionate-induced differentiation of adipocytes was mediated by GPCR43. As GPCR43 has been shown to mediate

differentiation and there was a trend of up-regulation of this receptor in HT29 cells with increasing confluence, which is also linked to differentiation, it was of great interest to determine if HT29 cells underwent differentiation with increasing cell confluence.

The results from the experiments in this Chapter showed no change in the differentiation status of HT29 cells with increasing cell confluency (Figure 6.5). These results suggest that the trend for increased GPCR43 expression observed at 80-90% confluence was not related to HT29 differentiation. Although other groups have looked at GPCR43 and differentiation, and shown a relationship, these groups investigated differentiation using different methodology, and in particular, differentiation which was induced by an external agent (Senga et al., 2003) (Hong et al., 2005).

6.5.4 Differentiation is induced following 5mM BuA exposure

In a similar way to the studies by Senga et al (Senga et al., 2003) and Hong et al (Hong et al., 2005), in the current study, the differentiation of HT29 cells using an external agent, in this case BuA, was also examined. The results from these experiments showed an increase in the differentiation status of HT29 cells following 5mM BuA exposure for 48 hours. This result is similar to many published studies, as described in Chapter 1.

Following the observation of increased differentiation with BuA exposure along with the BuA-induced changes to proliferation and apoptosis seen in Chapter 3, it was of great interest to measure the expression of GPCR43 following exposure to BuA. This was conducted in the following Chapter.

6.5.5 Other possible GPCR43 functions

Although the current study did not link the trend for increased GPCR43 expression to the physiological function of differentiation, it is still possible that an increase in receptor expression is linked to another function. The photographs in Figure 6.1 and 6.2 show distinct changes in cell morphology with increasing cell confluence, and may involve alterations in GPCR43 expression. It would be of great interest to identify why GPCR43 expression is increased with changes to cell confluence, through further investigation into the possible physiological functions that may be related to this interesting observation.

One potentially vital function of GPCR43 was hypothesised from the findings in the current Chapter. As cells grow in culture, proliferate and reach confluence they utilise glucose and other energy sources, which leads to a decrease in available nutrients in the media. As a result of decreased energy availability, it is possible the trend for the increase in GPCR43 expression at 80-90% confluence is a metabolic response of the cells to obtain energy from other sources such as BuA. The possibility that altered GPCR43 expression changes following changes in nutrient availability, and the possible role of GPCR43 as a BuA sensor, was investigated in detail in the next Chapter.

6.5.6 Summary

The results from this section show a large but insignificant increase in GPCR43 expression is observed at 80-90% confluence and therefore to ensure consistency it is vital to test the expression of this receptor at approximately 85% confluence. Furthermore, this study has demonstrated that the trend for an increased expression of GPCR43 at 80-90% confluence is not related to an increase in HT29 cell differentiation. On the other hand, 5mM BuA is sufficient to induce differentiation of HT29 cells, and therefore it was of great interest to investigate the expression of GPCR43 following 5mM BuA exposure. This relationship has been studied in the next Chapter.

7 Modulation of GPCR43 expression with BuA treatment and exposure to alternate energy sources in BuA-sensitive and BuA-resistant CRC cells

7.1 Introduction

BuA is the major source of energy for the colonic epithelium *in vivo* (Roediger, 1980) (Singh et al., 1997) and in rats it has been demonstrated to account for approximately 70% of total energy consumed by colonocytes (Scheppach, 1994). As described in Chapter 1, BuA has been demonstrated to promote proliferation in *in vivo* studies (Scheppach et al., 1992) whilst in *in vitro* studies using CRC cell lines, BuA decreases cell proliferation (Tsao et al., 1983) (Augeron and Laboisse, 1984). Singh et al (Singh et al., 1997) proposed that the apparent contrast in roles of BuA *in vivo* compared to *in vitro* can be accounted for by the availability of high concentrations of glucose to cells grown *in vitro*. Despite BuA being the preferred energy source of colonic epithelium *in vivo* most cell cultures are grown in medium containing relatively high levels of glucose (Singh et al., 1997). They reported that the effects of BuA on HT29 cell proliferation and apoptosis are dependent on alternative energy sources in the media by demonstrating that the effects of BuA are different when glucose and pyruvate are removed from the media (Singh et al., 1997).

As discussed in Chapter 1, it has been proposed that a switch from 'BuA-driven' to 'glucose-driven' metabolism occurs with cancer transformation (Boren et al., 2003) (Lambert et al., 2002) by cells up- or down-regulating the expression of BuA and glucose transporters following a change in the availability of nutrients in the media (Cuff et al., 2002) (Lambert et al., 2002) (Li et al., 2006). The concentration of BuA and other nutrients in the colon also varies markedly depending on diet, and the ability of colonic epithelial cells to sense and consequently adapt to the constantly fluctuating dietary nutrient concentrations is essential for survival (Dyer et al., 2003b). GPCRs have been implicated in nutrient sensing in the gastrointestinal tract and GPCR40, a member of the same family as GPCR43, has been shown to act as a FA sensor and amplify glucose induced insulin release (Itoh et al., 2003) (Itoh and Hinuma, 2005) (Poitout, 2003). The current study examined the possibility that GPCR43 is acting in the colon as a BuA sensor. To investigate the possible role of GPCR43 in BuA sensing, expression of the receptor was examined with BuA exposure in the presence of high or low glucose media. Both HT29 and HCT116 cells were used to investigate the effect of 5mM BuA

exposure on the expression of GPCR43, as HT29 cells are representative of early stage disease and HCT116 cells are representative of late stage CRC. Although GPCR43 expression was not detectable in HCT116 cells in Chapter 4 and 5, this cell line was included in this experiment to determine if exposure to BuA could induce expression. The proliferative and apoptotic responses of HT29 cells grown in high and low glucose media were also investigated.

In order to examine changes induced in cells exposed to high and low glucose media, the compositions of the media were altered. At the time of this experimentation, only glucose-free DMEM was available. Therefore, HT29 cell media which is comprised of 1:1 DMEM and F-12 was reduced to 30% of normal glucose concentrations compared with the normal high glucose media. The media composition was termed “low glucose media”. As McCoy’s media without glucose was not available commercially, HCT116 cells could not be examined in high and low glucose experiments.

In addition to changes in the preferential energy source of CRC cells, the acquisition of resistance to apoptotic or chemotherapeutic drugs is a major obstacle for cancer treatment (Olmo et al., 2007). Many solid tumours, including CRC, can undergo a number of cellular processes which enable the development of resistance to chemotherapeutic agents (Cusack, 2003). Following exposure to selection forces, established cancer cells with a survival advantage can be “selected for”, thus creating a population of cells with an acquired resistance mutation (Cusack, 2003). CRC cells can become resistant to BuA and apoptosis, and this may lead to the accumulation of cells with significant DNA damage potentially leading to malignant transformation (Rupnarain et al., 2004). It has been reported that some cell lines are more resistant to BuA and the results from Chapter 3 in this study have also shown differences in the sensitivity of cell lines to BuA. As BuA has been demonstrated to inhibit the development of CRC, understanding the mechanisms underlying resistance to BuA may also provide insight into the development and progression of CRC in patients. To investigate the potential role of GPCR43 in the resistance of CRC cells to BuA-induced apoptosis, analysis of a HT29 butyrate-resistant cell line (HT29-R) was also conducted.

HT29-R cells were established in the current laboratory by growing HT29 cells in the presence of BuA (Chalkley and Shires, 1985) (Olmo et al., 2007) (Lopez de Silanes et al., 2004). To adapt cells to growth in the presence of BuA, increasing concentrations of BuA in 0.5mM increments were added until the desired concentration of BuA was reached. This method is described in detail in the Materials and methods section 2.1

and is based on the paper published by Chalkley and Shires in 1985 (Chalkley and Shires, 1985). The level of GPCR43 expression along with the apoptotic and proliferative response to BuA exposure was investigated in this cell line.

7.2 Approach

1. To investigate the expression of GPCR43 and the corresponding changes to apoptosis and proliferation in HT29 and HCT116 cells with varying butyrate and glucose concentrations.
2. To measure the expression of GPCR43 and the proliferative and apoptotic response of HT29-R cells following exposure to varying concentrations of BuA.

7.3 Methods

7.3.1 Methods for investigating the morphological changes to CRC cell lines following changes to BuA and glucose concentrations

Photographs of HT29, HCT116 and HT29-R cells in the presence and absence of BuA were taken as described in the methods section 2.5.1. This enabled any morphological changes that may have occurred with the different glucose and BuA concentrations in the media to be identified.

7.3.2 Methods for determining expression of GPCR43 in the presence and absence of 5mM BuA

Analysis of GPCR43 expression was conducted in the presence and absence of BuA for 24 and 48 hours in both HT29 and HCT116 cells. T_{75} flasks with cells of approximately 85% confluence were treated with trypsin, plated at equal seeding density (1:4 split) into T_{75} flasks and incubated for 24 hours at 37°C, 5%CO_2. After 24 hours, media was removed and replaced with normal growth media with or without 5mM BuA and the flasks subsequently incubated at 37°C, 5% CO_2 for either 24 hours or 48 hours. RNA was then extracted and processed for Q-PCR as previously described in 2.2.2-2.3.1 and 2.4.1.

7.3.3 Methods for determining expression of GPCR43 in the presence and absence of 5mM BuA in high or low glucose media

HT29 medium was prepared as described in Chapter 2 using glucose-free DMEM (Invitrogen, Aust #11966-025). As HT29 cell medium has a 50% DMEM component,

normal HT29 media, made with high glucose DMEM, was termed high glucose (25mM). Media made with glucose-free DMEM contained approximately 30% less glucose and was termed low glucose. The expression of GPCR43 was examined in the presence of media containing high and low glucose as using the methods described in 2.2.2-2.3.1 and 2.4.1.

7.3.4 Methods for determining expression of GPCR43 in HT29-R cells grown in high or low glucose media

HT29-R cells were generated and cultured according to 2.1. T_{75} flasks of approximately 85% confluence were treated with trypsin and plated at equal seeding density (1:4 split) and incubated for 24 hours 37°C, 5%CO_2. After 24 hours, media was removed and flasks were replaced with either high or low glucose media containing 5mM BuA. Cells were incubated at 37°C, 5%CO_2 until 85% confluence was reached and the expression of GPCR43 assessed according to methods described in 2.2.2-2.3.1 and 2.4.1. Cells were grown to 85% confluence as a large but insignificant difference between the expression of GPCR43 was observed with changes in cell confluence, as described in the previous Chapter. Growing HT29-R cells to 85% confluence, took approx 5 days instead of the 48 hours it would take normal HT29 cells plated at that density to grow to 85% confluence. However, by having both the HT29-R and HT29 cells at 85% confluence it enabled the copies of GPCR43/μg total RNA to be directly compared between the HT29-R cells and the HT29 cells grown in Chapter 5 and HT29 cells grown in high glucose media alone for 48 hours in the current Chapter.

7.3.5 Proliferation and Apoptosis of HT29-R cells in the presence BuA

HT29-R cells were used for this experimentation using the methodology described in section 2.6.1. A kind thank you is extended to Dr Tanya Lewanowitsch for conducting these experiments.

7.3.6 Proliferation and Apoptosis in the presence BuA and media containing high or low glucose

Cell were plated according to Chapter 2 and incubated for 24 hours. After this seeding time, media was aspirated and BuA concentrations ranging from 1mM to 20mM diluted into culture media containing either high or low glucose were added to each of the wells. Plates were then incubated and proliferation and apoptosis was measured as described in 2.6.1.

7.3.7 Statistical analysis

All analyses were conducted using One-Way ANOVAs performed with Tukey's post hoc testing and significance indicated by values $p<0.05$. All statistical analyses were conducted using GraphPad Prism 5 software for Windows.

7.4 Results

7.4.1 Changes to cell morphology

7.4.1.1 HT29 and HCT116 cells exposed to 5mM BuA

Untreated HT29 cells appear rounded after growth in media containing no BuA for 24 hours (Figure 7.1). After 48 hours the cells have a flattened out appearance and adopted a more epithelial like phenotype with rounded edges. Untreated HCT116 cells at both time points were similar to the epithelial type cells described in Vecsey-Semjen et al, however they have hexagonal or 'sharp edges' (Figure 7.2).

HT29 cells treated with BuA for 24 and 48 hours showed a reduced cell size and appeared to be elongated with sharp edges (Figure 7.1). HCT116 cells also showed a greatly reduced cell size and had a rounded up morphology following 5mM BuA exposure for 24 or 48 hours (Figure 7.2).

7.4.1.2 HT29 cells exposed to 5mM BuA in media containing high and low glucose concentrations

In response to 5mM BuA exposure, the morphology of HT29 cells in this experiment showed highly changes to those described above in both high and low glucose (Figure 7.3a-b). No obvious changes in morphology were detected between the high and low glucose group over this treatment time.

7.4.1.3 HT29-R cells exposed to media containing high and low glucose concentrations

HT29-R cells showed an epithelial like morphology which was not changed with alterations to the media glucose concentration (Figure 7.4).

7.4.2 Changes in GPCR43 expression following exposure to 5mM BuA

The copies/μg total RNA for HT29 cells at 0mM BuA was 6152 ± 1588 and 9596 ± 1921 for 24 and 48 hours respectively. In this cell line the expression of GPCR43 was

significantly reduced following exposure to 5mM BuA for 24 and 48 hours respectively (Figure 7.5). HCT116 cells were demonstrated previously in Chapter 4 and 5 to be negative for GPCR43 expression. The results from the present experimentation further demonstrates that the addition of 5mM BuA to HCT116 cells had no influence on the absence of GPCR43 expression in this cell line (Figure 7.6).

Uniform expression of 18S was observed in both HT29 cells and HCT116 cells at all concentrations of BuA and time points tested, Figure 7.5b and 7.6b respectively. The no template controls were negative for amplification, and indicated no contamination had occurred. These results indicate that the Q-PCR reactions were successful and validates the observations for GPCR43 expression.

7.4.3 Changes in GPCR43 expression in HT29 cells following exposure to BuA in high or low glucose media

As shown previously the expression of GPCR43 in HT29 cells grown in high glucose media was significantly decreased with the addition of 5mM BuA after 24 and 48 hours. In low glucose media, GPCR43 expression was also significantly decreased with the addition of 5mM BuA after 24 and 48 hours respectively (Figure 7.7). Interestingly, at both the 24 and 48 hour time points, the expression of GPCR43 tended to be higher in low glucose media compared to high glucose media in the absence of BuA with this difference achieving significance at the 48 hour time ($p < 0.05$).

Uniform expression of 18S occurred in all treatment groups as summarised in Figure 7.8.

7.4.4 Alterations to HT29 proliferation and apoptosis following exposure to 5mM BuA in high or low glucose media

7.4.4.1 Proliferation

At the 24 hour time point, there was no significant effect of BuA on the proliferation of HT29 cells grown in high glucose media (Figure 7.9a). Cells grown in low glucose media alone (0mM BuA) exhibited significantly less proliferation ($p<0.001$) when compared to high glucose media alone (0mM BuA). With the addition of 2.5mM BuA to cells in low glucose media, a significant increase in proliferation was observed. This increase in proliferation remained constant with increasing BuA concentrations up to 20mM.

At the 48 hour time point, HT29 cell proliferation decreased with increasing BuA concentrations in high glucose media (Figure 7.9b). This occurs in a dose-dependent manner comparable to the observation in Chapter 3. In the absence of BuA, cell proliferation is significantly lower in media containing low glucose compared to high glucose. In low glucose media, the addition of 2.5mM and 5mM BuA enhanced the proliferation compared to low glucose media alone. At 7.5mM BuA, proliferation in the low glucose media began to decrease, and continued to decrease with increasing BuA concentrations.

7.4.4.2 Apoptosis

After 24 hours, there was no difference in the degree of apoptosis between HT29 cells grown in high glucose media alone (0mM BuA) compared to low glucose media alone (Figure 7.10a). The addition of 2.5mM BuA is sufficient to significantly increase apoptosis in cells grown in high glucose media alone, but not in cells grown in low glucose media. Higher concentrations of BuA at this time point caused a dose-dependent increase in apoptosis in cells exposed to both high and low glucose media.

After 48 hours, a dose-dependent BuA-induced apoptotic response of HT29 cells was observed in both high and low glucose media (Figure 7.10b). There was no significant difference in BuA-induced apoptosis between cells grown in high and low glucose media at 0-15mM BuA. In HT29 cells treated with 20mM BuA, significantly higher apoptosis was observed in low glucose media compared to high glucose media. It is also worth noting that the magnitude of apoptotic response at 48 hours was much greater than that observed at 24 hours (Figure 7.10).

7.4.5 Changes in GPCR43 expression of HT29-R cells in high or low glucose media

The values obtained for GPCR43 expression in HT29-R cells were comparable to the values observed for the HT29 cells exposed to BuA for 48 hours in both high and low glucose media (Table 7.1).

	High Glucose Media	Low Glucose Media
HT29 with 48 hour BuA exposure	2441 ± 353	2463 ± 714
HT29 Resistant cells	2363 ± 178	2248 ± 192

Table 7.1: Expression levels of GPCR43 in HT29 cells exposed to BuA and HT29-R cells grown in high and low glucose conditions.

Expression of GPCR43 in HT29-R cells is not significantly altered by the glucose content of the media (Figure 7.11a). Consistent expression of 18S is observed in both samples and is shown in Figure 7.11b.

7.4.6 Changes in proliferation and apoptosis of HT29-R cells in high glucose media

7.4.6.1 Proliferation

Normal HT29 cells showed decreased proliferation following exposure to BuA in a similar manner to the results previously described in this Chapter and Chapter 3. There is no significant change to the proliferative response of HT29-R cells exposed to any concentration of BuA for 24 or 48 hours (Figure 7.12). In the normal HT29 cells, the BuA-induced proliferative response at 24 and 48 hours was similar to that previously described in Chapter 3. Although the concentrations of BuA tested in this experiment are different to experiments described in previous Chapters, a similar dose response pattern is observed.

7.4.6.2 Apoptosis

Normal HT29 cells showed increases in apoptosis following exposure to BuA in a similar manner to the results previously described in this Chapter and Chapter 3. HT29-R cells showed minor increases in apoptosis following exposure to 25mM and 50mM BuA, but these were not statistically significant (Figure 7.13). A similar result was observed in the same cell line at 48 hours, with small, non significant increases in apoptosis observed again at with 25mM and 50mM BuA.

7.5 Discussion

7.5.1 Changes in cell morphology following BuA exposure

7.5.1.1 HT29 and HCT116 cells exposed to 5mM BuA

The addition of 5mM BuA showed a striking change in cell morphology for both HT29 and HCT116 cells after both 24 and 48 hours (Figure 7.1 and 7.2). HT29 and HCT116 cells treated with BuA for both 24 and 48 hours show a reduced cell size after 24 and 48 hours (Figure 7.1-7.2). The treated HT29 cells appeared to be elongated with sharp edges (Figure 7.1). These changes in cell morphology are similar to changes observed

using the human non-small lung carcinoma cell line H460 exposed to BuA as described by Joseph et al (Joseph et al., 2004). HCT116 cells have a rounded up phenotype following 5mM BuA exposure for 24 or 48 hours (Figure 7.2). A similar result was also observed in the rat colon carcinoma cell line PROb by Bonnotte et al (Bonnotte et al., 1998) following exposure to BuA and Fas-ligand. Collectively, this experimentation demonstrates a significant influence of BuA on cell morphology.

After incubation for 48 hours fewer adherent cells were present and more floating cells were observed in the BuA treated compared to untreated cells. Increased cell death via apoptosis leads to detachment of cells and causes the increase in the floating cell number. This phenomenon is described in many papers (Hague et al., 1995; Hague et al., 1993; Hague and Paraskeva, 1995) (Singh et al., 1997). However, it must be acknowledged that changes to cell morphology were not quantified using image analysis. It would be important in future experimentation to conduct further quantitative studies to determine the exact extent of change to cell morphology.

7.5.1.2 The morphology of HT29 cells exposed to 5mM BuA is not modified by high or low glucose treatments

Like the HT29 cells described in the section above (7.5.1.1), 5mM BuA in high and low glucose media induced changes to cell morphology. However, no visual differences in cell morphology were detected between the high and low glucose media treatments in either the BuA-free media or media containing 5mM BuA (Figure 7.3a-b). The results from the current study are similar to the report published by Stokrova et al (Stokrova et al., 2005). Using electron microscopy, these researchers demonstrated that BuA-induced differentiation or apoptosis in the human colon carcinoma HCT115 cell line was similar when comparing cells deprived of glucose to cells exposed to glucose.

7.5.2 HT29-R cells exposed to media containing high and low glucose concentrations

7.5.2.1 Changes to cell morphology

HT29-R cells have an epithelial-like morphology which did not appear to change with alterations to the glucose concentration in the media (Figure 7.4). It is intriguing to observe that HT29-R cells that have been subjected to chronic BuA exposure over a time frame greater than 6 months have an appearance closely resembling untreated HT29 cells rather than HT29 cells which have had acute exposure to 5mM BuA. This

result indicates that BuA treatment initially induces a dramatic change in cell morphology and that the acquisition of BuA resistance in HT29 cells is accompanied by a “reversion” in the morphology such that HT29-R cells look like untreated HT29 cells.

7.5.3 Changes to GPCR43 expression with BuA treatment

GPCR43 expression in HCT116 cells was previously shown to be undetectable using Q-PCR (Chapter 5). The absence of GPCR43 expression in HCT116 cells was not altered by exposure to 5mM BuA (Figure 7.6a). To test the cDNA integrity, expression of the housekeeping gene 18S was conducted, and consistent expression was observed in HCT116 cells, thus validating these results (Figure 7.6b). Furthermore, the expression of 18S expression in HT29 cells in all treatment conditions was also even and uniform cDNA synthesis occurred across all samples (Figure 7.5b). This finding confirms the earlier findings (Chapter 5) that GPCR43 is not expressed on HCT116 cells and also indicates that the absence of GPCR43 expression is not influenced by BuA treatment.

The expression of GPCR43 in HT29 cells was significantly decreased following 5mM BuA exposure for 24 and 48 hours in both high and low glucose media (Figure 7.7). It has been demonstrated that the exposure of GPCRs to ligand often results in the rapid reduction of receptor sensitivity, and is termed desensitisation (Ferguson, 2001). This phenomenon acts as a negative feedback mechanism to prevent both chronic and acute receptor over-stimulation (Ferguson, 2001). Receptor over-stimulation is often observed in many disease states, including cancer (Heasley, 2001) (Horn et al., 2000). Desensitisation of GPCRs occurs through multiple mechanisms including the uncoupling of the receptor from the G-protein subunits (Bouvier et al., 1988) , internalisation of existing receptors (Hermans et al., 1997) (Trejo et al., 1998), and the down-regulation of receptor expression via reduced mRNA and protein synthesis (Hadcock and Malbon, 1988) (Ferguson, 2001). The time required for each of these processes ranges from seconds (uncoupling events) to minutes (internalisation) or hours (down-regulation events) (Ferguson, 2001). It is probable that the prolonged exposure of GPCR43 to BuA, one of its ligands, lead to receptor desensitisation and subsequent mRNA down-regulation. This would account for the decrease in GPCR43 expression following exposure to BuA observed in this Chapter.

7.5.4 Changes in GPCR43 expression in high and low glucose media with the addition of 5mM BuA

Bruce et al (Bruce et al., 2000) proposed a mechanism to explain how high glucose concentrations may occur in colon tissue as a result of an excessive diet. This paper describes how excessive consumption of food could lead to increased availability of glucose and other energy substrates to the colon, and how the excess energy in the colon and other organs may lead to an increased risk of carcinogenesis (as discussed in Chapter 1 and shown in Figure 1.7). Energy is usually consumed by muscle and liver, with any excess energy being transformed to adipose tissue. In a Western diet there is often an energy imbalance, with excessive energy being consumed. If an excessive diet is consumed for a prolonged period of time, the tissues that normally consume the energy become insulin-resistant and the colon and other organs are then exposed to higher levels of glucose, free fatty acids and triglycerides. Excess glucose in the colon is likely to induce a number of significant modifications, and as described in Chapter 1, may change the expression of molecular energy transporters as a result of higher availability of glucose in the absence of BuA. As described in Chapter 1, the transition from BuA-driven metabolism to glucose-driven metabolism and the corresponding modifications to energy transport systems is likely to be a key initiator in the development of CRC (Jass, 1985), with a number of studies demonstrating changes to transporters correlated with the transition from normalcy to malignancy. Lambert et al (Lambert et al., 2002) demonstrated that MCT1 expression is decreased with the progression of cancer as well as an up-regulation of the high affinity Glut 1 transporter coupled with a down-regulation of the low affinity Glut 2 transporter. Other papers which have described similar results have been outlined in Chapter 1.

In the current study the expression of GPCR43 in the presence of high and low glucose media with or without BuA was investigated to identify the role that GPCR43 may play in a potential switch from BuA-driven to glucose-driven metabolism. In the absence of BuA, GPCR43 expression tended to be higher in HT29 cells grown in low glucose media, compared to high glucose media (Figure 6A), with the observation reaching significance after 48 hours. Previous studies have observed similar results by demonstrating a reversible adaptation of HT29 cells to different nutrient environments (Boren et al., 2003). This occurs via changes in expression levels of glucose transporters (Li et al., 2006) and BuA transporters (Cuff et al., 2002) in response to an altered nutrient environment. An up-regulation of the BuA transporter MCT1 (Cuff et al., 2002) and a down-regulation in Glut1 transporters (Li et al., 2006) was demonstrated in

media supplemented with BuA. Both Li et al (Li et al., 2006) and Cuff et al (Cuff et al., 2005) suggested that the changes in glucose and BuA transporters respectively occurred as a response to changes in the availability of different metabolic substrates in the media. In the current study, it is possible that the up-regulation of the GPCR43 receptor expression in media containing low glucose concentrations (Figure 7.7) is a metabolic response of the cells arising from the reduced availability of an energy source. Due to the decreased glucose concentration, it is possible that there will be an up-regulation of sensors or transporters which have the potential to detect or supply energy from glucose or alternate energy sources may occur.

The hypothesis that GPCR43 acts as a BuA sensor is strengthened by the increased receptor expression in low glucose media alone compared to high glucose media alone. It would be of interest to determine if the same trend is observed in completely glucose-free media. This could be performed by either sourcing glucose-free F-12 media to combine into the HT29 media currently used, or growing the HT29 cells in glucose-free DMEM media as conducted by other studies (Singh et al., 1997).

In addition, the observation that GPCR43 expression was decreased following acute BuA exposure (summarised in Figure 7.5a and 7.7) further supports the concept that GPCR43 may act as a sensor for fluctuating concentrations of BuA and other SCFAs in the colon. The results showed that the addition of 5mM BuA to both high and low glucose media caused a decrease in GPCR43 receptor expression over the 24 and 48 hour period examined in this experiment. As described earlier in this Chapter, it is possible that extracellular BuA caused over-stimulation of GPCR43 receptors and subsequent down-regulation of the receptor.

Expression of GPCR43 was tested at 24 and 48 hours to correlate expression data with observable changes in apoptosis and proliferation at the 24 and 48 hour time points. However, Ascoli et al (Ascoli and Puett, 1978) used radioactive ligand binding techniques to show a decrease in the number of β-Adrenergic receptors, another type of GPCR, following just 1-6 hour exposure to an agonist. Therefore, more comprehensive testing of GPCR43 expression at various time points starting within the 1-6 hours, as described by Ascoli et al (Ascoli and Puett, 1978), may provide further information regarding the BuA-induced changes to GPCR43 expression.

7.5.5 BuA-induced proliferation and apoptosis of HT29 cells grown in high or low glucose media.

7.5.5.1 Proliferation

HT29 cells were exposed to BuA for 24 or 48 hours in the presence of high and low glucose concentrations. After 24 hours of BuA treatment in high glucose media, HT29 cells showed no significant change in proliferation (Figure 7.9). However, after 48 hours, HT29 cells displayed a dose-dependent decrease in proliferation (Figure 7.9) similar to that observed in Chapter 3. In media containing low concentrations of glucose, a somewhat different proliferative profile was observed. The first notable difference was that in the absence of BuA (0mM) the proliferation of cells grown in low glucose was lower than the cells grown in high glucose media at both 24 and 48 hours. This observation is not unexpected as the cells have much lower energy availability in the low glucose conditions. A similar effect was observed by Singh and colleagues (Singh et al., 1997) when they investigated the effects of BuA exposure on HT29 cells grown in media with or without glucose and pyruvate (Glu^+Pyr^+ or Glu^-Pyr^-). They examined cell growth by measuring the number of cells attached to the flask after treatment, and demonstrated that in (Glu^+Pyr^+) media approximately 19 x 10^6 cells were attached at the 0mM BuA concentration compared to approximately 6 x 10^6 cells attached in the (Glu^-Pyr^-) media. The decrease in cell proliferation in the absence of glucose was more pronounced in the experiments conducted by Singh et al (Singh et al., 1997) than in the current study, although, this may be largely accounted for by the difference in the constituents of the normal growth media. Singh (Singh et al., 1997) used glucose and pyruvate-free DMEM media which contained no alternate energy sources, whereas in the current study the low glucose media still contained glucose and pyruvate. Furthermore, in the study by Singh (Singh et al., 1997) the cells were incubated in the various concentrations of BuA, pyruvate and glucose for 4 days prior to analysis, whereas in the present study this analysis was completed after 48 hours.

The second notable and somewhat intriguing observation in the current study was that HT29 cells grown in low glucose media alone (0mM BuA) have a significant increase in proliferation following exposure to 2.5mM BuA at the 24 hour time point to levels similar to those observed in the high glucose treatment. At the 48 hour time point, 2.5mM-5mM BuA increased proliferation of HT29 cells grown in high glucose media. As described, Boren et al (Boren et al., 2003) demonstrated a reversible adaptation of HT29 cells to different nutrient environments, and showed that a switch from glucose-driven metabolism to BuA-driven metabolism occurred depending on the energy source

available. It is tempting to speculate that the BuA-induced increase in cell proliferation of HT29 cells grown in low glucose media is a part of the reversion of HT29 cells to a BuA-driven metabolism when high glucose concentrations are not available to be utilised for energy. This concept will be discussed in more detail in the upcoming section.

The increase in proliferation induced by 2.5mM BuA in low glucose media remained consistent despite the addition of increasing concentrations of BuA (5-20mM) at the 24 hour time point (Figure 7.9). In a similar way, the addition of increasing concentrations of BuA to cells grown in high glucose media did not affect cell proliferation after 24 hours. These results indicate that the HT29 cells were not adversely affected by BuA exposure in for at least 24 hours irrespective of glucose concentration. This observation may be explained by the hypothesis presented by Jass (Jass, 1985) as described in Chapter 1. Jass proposed that that the shift in metabolism occurring with the progression of colorectal carcinogenesis would interfere with the metabolism of BuA, leading to an accumulation of BuA within the cell cytoplasm and eventual toxicity. Applying this hypothesis to the results of the current study, it can be speculated that HT29 cells can metabolise BuA effectively for at least 24 hours before there is an accumulation of BuA that is detrimental to the cells. This may explain why following 48 hours exposure, there is a decreased proliferation of HT29 cells in both high and low glucose following BuA exposure as seen in Figure 7.9.

7.5.5.2 Apoptosis

The results from this Chapter also demonstrated that the apoptotic response of HT29 cells to increasing concentrations of BuA is not markedly changed with high and low glucose concentrations after 24 and 48 hour time points (Figure 7.10). In media alone (0mM BuA), there is no significant difference in apoptosis between cells grown in high or low glucose media. Singh et al (Singh et al., 1997) demonstrated slightly higher levels of apoptosis in low glucose media alone compared to high glucose alone. The differences between these two studies could be accounted for in part by the differences in cell media and experimental design as highlighted earlier in this Chapter in section 7.5.5.1 and in Chapter 3.

At the 24 hour time point, 2.5mM BuA is sufficient to significantly increase apoptosis of HT29 cells grown in high glucose media, but 7.5mM BuA must be added to HT29 cells grown in low glucose media to detect significant increases in apoptosis. Despite this,

only the apoptosis at 2.5mM BuA between high and low glucose showed statistical significance. The results from this section may suggest that in low glucose media there is a reversion of the cell to a BuA-driven metabolism, as described in Boren et al (Boren et al., 2003) and discussed above.

Following 48 hour exposure, the magnitude of the apoptotic response was increased from that observed after 24 hours. Cells grown in high glucose media exhibited increased apoptosis at this time point in the same dose-dependent manner observed in Chapter 3. In the low glucose media, observable increases in apoptosis were detected with 5mM BuA and became statistically significant with 7.5-20mM BuA. There are minimal differences between the apoptotic response of HT29 cells grown in high and low glucose media, except for significantly higher levels of BuA-induced apoptosis occurring in low glucose media compared to high glucose media with the addition of 20mM BuA (Figure 7.10). Although studies have described 20mM as an acceptable physiological concentration of BuA in the colon, most *in vitro* experiments utilise concentrations much lower than this, typically 5mM. Therefore the 20mM concentration of BuA would be considered to be very high for an *in vitro* study, and as no other differences were observed between high and low glucose at any other BuA concentration, it is difficult to draw conclusions that may be of relevance to the physiological state.

Singh et al (Singh et al., 1997) demonstrated high levels of BuA-induced apoptosis in HT29 cells grown in high glucose media compared to low glucose media, however that study only used a maximum BuA concentration of 6mM. In the current study, 5mM and 7.5mM BuA produced no observable differences between high and low glucose conditions. As previously described, experimental differences and the methods used to measure apoptosis may account for these differences between studies. Conversely, like the present study, Li et al (Li et al., 2006) demonstrated that higher levels of BuA-induced apoptosis occurred in low glucose media.

7.5.5.3 Summary of HT29 response to 24 and 48 hour BuA exposure

Despite the obvious experimental differences between the study by Singh et al (Singh et al., 1997) and the current study, the HT29 response to BuA in media with or without other energy sources is largely consistent. The proliferation and apoptosis results from this section may suggest that cancer cells are able to sense a low glucose, high BuA environment and consequently adapt to the extracellular environment and utilise BuA as an energy source. A similar conclusion was made by Boren et al (Boren et al., 2003)

and Lambert et al (Lambert et al., 2002) who showed that HT29 cells exposed to BuA were able to re-adapt to the high BuA environment by changing the expression of energy transporters. Singh et al (Singh et al., 1997) also demonstrated that [^{14}C]butyrate was more rapidly metabolised in glucose and pyruvate free media ($Glu^{-}Pyr^{-}$), and also provided evidence that BuA was used as an energy source in the absence of other nutrients. Earlier in this Chapter, it was demonstrated that the expression of GPCR43 was altered in different glucose and BuA conditions, potentially indicating a role in BuA metabolism.

7.5.6 Changes in expression of GPCR43 on HT29-R cells

As mentioned in the Introduction of this Chapter the acquisition of resistance to chemotherapeutic agents in CRC cells is a major problem in the treatment of cancer (Olmo et al., 2007). The ability to devise treatments strategies that overcome resistance to chemotherapeutic agents is one of the most challenging obstacles to successfully treating patients with metastatic CRC (Schmitt and Lowe, 1999). To investigate the potential role of GPCR43 in the acquisition of BuA resistance, the expression of GPCR43 was investigated in HT29-R cells along with the proliferative and apoptotic response of these cells in response to BuA exposure.

When measuring the expression of GPCR43 in HT29-R, cells it was of greatest interest to compare these results to the “parent” HT29 cells, which have previously been shown to be highly responsive to BuA treatment. However, in the current study HT29-R cells have significantly slower growth, a phenomenon also noted by Chalkley and Shires (Chalkley and Shires, 1985) and by Lopez de Silanes et al (Lopez de Silanes et al., 2004). The expression of GPCR43 was shown to be increased (although not significantly) by changes in confluence in Chapter 6. Therefore, although the increase in GPCR43 expression with increased cell confluence was not significant, it was considered important to ensure that the HT29-R cells were grown to 85% confluence in the same manner as HT29 cells from Chapter 5 to allow comparisons between the 2 cell lines. This also means that the expression of GPCR43 in high glucose media alone for 48 hours can be used in comparisons against HT29-R cells as both have 85% confluence.

Using the HT29-R cells, comparisons between the effect of chronic and acute exposure to BuA could also be investigated. However, it must be noted that in these instances confluency of each group could not be maintained as the treatment of cells with an acute

dose of BuA induced significant cell death and consequently affected confluency. To ensure that seeding density and treatment times for both BuA treated and untreated cells was consistent it was unfortunately impossible to have matched confluences in both groups at the end of this experiment. Despite this unavoidable limitation some interesting results were observed as discussed below.

The results from this section show that HT29-R cells expressed 2363 ± 178 and 2248 ± 192 copies of GPCR43/μg total RNA when grown in high or low glucose respectively (Figure 7.11a and Table 7.1). This value is substantially lower than the expression values obtained for HT29 cells in Chapter 3. Levels of expression of GPCR43 in HT29-R cells are comparable with the levels expressed in HT29 cells exposed to an acute dose of BuA. This study indicated that a similar level of expression of GPCR43 occurred with both chronic and acute BuA exposure. This result suggests that decreased GPCR43 expression levels do not adapt following continual BuA exposure, and may be a further indicator that GPCR43 is acting as a BuA sensor as in the presence of ligand (in this case BuA) the receptor continues to be down-regulated irrespective of the time of exposure.

7.5.7 Changes in proliferation and apoptosis of HT29-R cells in high glucose media

HT29-R cells had been growing the presence of 5mM BuA and it could be expected that they would be resistant to this concentration of BuA. However, it was of interest to determine whether higher concentrations of BuA, such as 50mM, could induce a reduction in proliferation and an increase in apoptosis. Thus higher concentrations of BuA well outside the physiological range were investigated. Very little induction of apoptosis or reduction in proliferation occurred in either the HT29 or HT29-R cells following BuA exposure for 24 hours. Following 48 hours of BuA exposure a difference in the response of normal HT29 and HT29-R cells is clearly evident (Figure 7.12 and 7.13). The normal HT29 cells responded to BuA in a dose-responsive manner with decreased proliferation and increased apoptosis observed with increasing BuA concentrations. The HT29-R cells did not show any significant decreases in cell proliferation with the addition of BuA concentrations up to 50mM. The apoptotic response of the HT29-R cells was also vastly different to the HT29 cells with the resistant cells showing small, though not significant, increases in apoptosis following exposure to 25mM and 50mM BuA concentrations. It is possible that further analysis extracting the properties on the HT29-R cells at 48 hours may show that the increase in

apoptosis seen in HT29-R cells at the 48 hour time point is significant. However, it is stated in the technical section of GraphPad Prism that "all groups should be analysed at once using a One-way ANOVA, and then followed up with multiple comparisons post tests". Therefore, a One-way ANOVA was utilised in this experimentation as this type of statistical test can be used to analyse 3 or more sets of data, with Tukey's post hoc testing to determine any differences between data sets. These results indicate that although the induction of apoptosis in HT29-R cells after 48 hours may appear to have increases from the 0mM control; this change is relatively small compared with the increased BuA-induced apoptosis observed in normal HT29 cells.

These results indicate that even though the HT29-R cells have been exposed to 5mM BuA in culture, they are resistant to this and much higher BuA concentrations. This may have implications for the treatment of CRC as it suggests that if cells have acquired resistance to BuA they will not respond to larger doses being administered. It should be understood that the current experiments are all conducted *in vitro* and are based on repeated passages of cells in constant BuA, which may not occur *in vivo*. However, Boren and colleagues (Boren et al., 2003) suggest that the re-adaptation of cells to high BuA environments probably occurs via the alteration of genes associated with glucose and lipid metabolism. This concept could be investigated by removing BuA from the media of HT29-R cells in the same step-wise manner that it was added and then re-assessing the apoptotic and proliferative response of the reverted cells along with the expression levels of GPCR43. This is currently being investigated in our laboratory along with the changes in expression of genes using cDNA or protein microarrays.

7.5.8 The relationship between GPCR43 and BuA-induced changes to proliferation and apoptosis

Earlier in Chapter 3 and 4 it was observed that while GPCR43 was expressed in HT29 cells, and its agonist BuA induced changes to proliferation and apoptosis in these cells, the association did not apply for other CRC cells tested. This led to the conclusion that GPCR43 expression and BuA-induced decreases in proliferation and increases in apoptosis are not tightly linked in CRC cells. The results of the experiments described in this Chapter are consistent with this conclusion. In HT29 cells both acute and chronic (HT29-R) exposure to BuA reduces the expression of GPCR43. However, despite this commonality, HT29 cells show strong BuA-induced decreases in proliferation and increases in apoptosis, whereas HT29-R cells show diminished responses to BuA. This

provides further evidence that a simple relationship between GPCR43 and BuA-induced changes to proliferation and apoptosis does not exist.

7.5.9 Conclusion

The focus of this Chapter was upon potential modulation of GPCR43 expression with BuA exposure (acute and chronic) as well as exposure to alternate energy sources (high and low glucose). The results highlight at least 3 conclusions:

1. Acute BuA treatment induces a decrease in proliferation and increase in apoptosis in HT29 cells which is also associated with a change in cellular morphology. These responses are not dramatically changed with alterations in glucose exposure. Chronic BuA treatment (HT29-R cells) markedly depresses the proliferative and apoptotic effects of BuA and is not associated with major changes to cellular morphology. While changes in glucose may modify aspects of HT29 function these experiments indicate:
    a. A major role of BuA in regulating HT29 cellular function.
    b. Further evidence for a disassociation of GPCR43 expression and BuA-induced decreases in proliferation and increase in apoptosis.
2. Acute BuA treatment decreases GPCR43 expression in HT29 cells. Therefore, it must be concluded that at the same time that BuA decreases proliferation and increases apoptosis in HT29 cells it is also influencing GPCR43 expression.
3. While there are subtle changes in apoptosis and proliferation mediated by glucose in the presence and absence of BuA, the influence of BuA is by far more powerful. Glucose treatment however, does modulate GPCR43 expression in the absence of BuA.

7.5.10 Summary

The hypothesis from the current study is that GPCR43 in the colon acts in a similar manner to the unknown GPCR glucose sensor in the intestine (Dyer et al., 2003b) (Dyer et al., 2003a), or like GPCR40, to sense nutrient changes in the extracellular environment (Itoh and Hinuma, 2005; Itoh et al., 2003). Results from this Chapter demonstrate that the expression of GPCR43 is modulated with different BuA and glucose environments. Furthermore, the effects of BuA on proliferation and apoptosis were altered depending on alternate energy sources available. Studies which have previously described similar results attribute the difference in BuA-induced effects in high and low glucose conditions to a modulation of key genes involved in metabolism. As GPCR43 expression is modified with changes to glucose and BuA concentrations, it

is possible that GPCR43 is involved in modulating the effects of BuA. A review paper and an experimental paper both published late in 2006 by Covington et al (Covington et al., 2006) and Karaki et al (Karaki et al., 2006) also came to a similar conclusion, and suggested that GPCR40-43 are a family of nutrient sensors. The findings from this Chapter provided a strong basis for further investigation into the role of GPCR43. RNAi knockdown was used in the next Chapter to investigate the biological role of GPCR43 in BuA-induced changes to apoptosis, proliferation and differentiation.

8 Knockdown of GPCR43 using RNAi

8.1 Introduction

The experiments detailed so far provide evidence for the expression of GPCR43 on HT29 cells and little to no expression on other CRC tested. Collectively the data suggests GPCR43 activity is not definitively linked to BuA-induced apoptosis and proliferation. A possible way to further explore this relationship is to investigate the activity of GPCR43 using RNAi. RNAi is an exciting new molecular biology tool which can be used to elucidate the function genes, identify new drug targets and develop more specific therapeutics (Jackson et al., 2003). Since RNAi was first discovered in 1998 by Fire et al (Fire et al., 1998) it has rapidly developed into a widely applied technique (Geley and Muller, 2004). The exact mechanism, roles and functions of RNAi are still under investigation, however, for the purposes of the current study RNAi was employed as a functional genomics tool. The basic principles of RNAi is summarised in Figure 8.1 and will be described in the current Chapter along with the application of the technique to silence GPCR43 expression to determine the role of this receptor in BuA-induced apoptosis and proliferation.

In 1998, it was demonstrated that double stranded RNA (dsRNA) injected into the nematode initiated a sequence-specific degradation of mRNA in the cytoplasm (Fire et al., 1998). Since that time a number of groups have shown that RNAi is not just a phenomenon in nematodes, but rather a highly conserved process with an origin that appears before the divergence of plants and animals (Sharp, 2001) . The proposed model for the mechanism of RNAi consists of 2 distinct steps; the initiation phase and the execution phase (Geley and Muller, 2004). A pictorial representation of each phase can be seen in Figure 8.1. The initiation phase begins when dsRNA is digested by an enzyme, called Dicer (Harborth et al., 2003), to short interfering RNA (siRNA) molecules of 21-23 nucleotides in length. During the execution phase, the siRNA duplex molecules are unwound and incorporated into a large multiprotein complex (Geley and Muller, 2004) (Harborth et al., 2003). This complex is involved in a number of gene silencing activities and is called the RNA-induced silencing complex (RISC) (Geley and Muller, 2004). After the unwinding of the siRNA duplex to form a single stranded siRNA molecule, the RISC complex with the incorporated single stranded siRNA molecule can target and degrade mRNA molecules having the exact antisense sequence. Thus, the introduction of dsRNA to plants and insect cell lines for the specific knockdown of gene expression has been highly successful (Hammond et al., 2000) (Clemens et al., 2000).

In mammalian cells, dsRNA induces a potent interferon response, which acts as an antiviral defence mechanism (Stark et al., 1998). Consequently, no specific RNAi knockdown can be induced by dsRNA in mammalian cells. Elbashir et al (Elbashir et al., 2001) overcame this obstacle by demonstrating that siRNA molecules of 21nt in length added directly to the cell were able to induce significant and specific gene expression knockdown. This discovery bypassed the antiviral response and enabled the investigation of gene function using RNAi knockdown in mammalian cells.

Although the ligands for the GPCR40-43 family of receptors were first described in 2003 (Briscoe et al., 2003) (Brown et al., 2003) (Le Poul et al., 2003), the exact function of these receptors is still largely unknown. In the first papers published on this family of receptors much of the work consisted of receptor activation assays to ascertain the ligand-receptor relationships, as well as tissue expression array experiments to determine where the receptors were expressed. Only recently has further research been conducted to establish the function of this family of receptors. Elucidating the function of GPCR43 has been attempted by a small number of groups, and some interesting and exciting observations have been obtained. However, there is still no final conclusion regarding the exact biological role of GPCR43.

From the studies described in Chapter 1 a number of possibilities for the role(s) of GPCR43 have been proposed. BuA, which is a GPCR43 ligand, has a number of physiological functions within the colon including the induction of apoptosis and decreased proliferation of colonocytes/epithelial cells. To investigate the possible role of GPCR43 in BuA-induced changes to proliferation and apoptosis, siRNA knockdown of the receptor was conducted, and the BuA-induced proliferative and apoptotic responses of HT29 cells with GPCR43 knockdown were compared to those in normal HT29 cells.

8.2 Approach

1. To optimise the siRNA transfection of HT29 cells using the BLOCK-iT^{TM} Fluorescent Oligo (Block-iT) (Invitrogen, Aust.).
2. To use the RNAi knockdown technique to determine if BuA-induced decreases in proliferation and increases in apoptosis of HT29 cells is mediated via GPCR43.

8.3 Methods

HT29 cells were used in the knockdown experimentation conducted in this Chapter as they have routinely demonstrated high levels of GPCR43 expression and alterations in apoptosis and proliferation in response to BuA treatment.

8.3.1 Cell Culture

All cell culture experimentation was conducted according to 2.1, however for RNAi experiments cells were cultured in media without antibiotics as this has been shown to aid transfection/knockdown in a number of papers (Doench et al., 2003) (Liao et al., 2004) (Fedorov et al., 2006) (Palayoor et al., 2005).

8.3.2 Testing transfection of HT29 cells

To determine the transfection of siRNA molecules into HT29 cells a fluorescent oligo was utilised. When excited with light at a wavelength of 494 nm and emission detected at 519nm, cells with efficient uptake of the BLOCK-iT oligo fluoresce which can be observed using fluorescence microscopy.

8.3.2.1 Cell culture

Cells from 85% confluent T_{75} flasks were treated with trypsin, counted and 5000-50,000 cells were replated into clear 96-well plates (Nunc, Sweden) containing 100μl of media. Plates were then incubated at 37°C, 5% CO_2 for 24 hours.

8.3.2.2 Dilutions of siRNA oligos and transfection reagent

All solutions were made according to the manufacturer's instructions. Initially the BLOCK-iT siRNA oligos were diluted in RNAse-free water to yield a final concentration of 20μM and stored a -20°C. The 20μM stock was further diluted to a 2μM working stock immediately prior experimentation.

The individual volumes of each reaction have been presented in this section. However, due to the small volumes, master mixes of each solution were made at the time of experimentation to reduce pipetting error. The BLOCK-iT siRNA molecules were diluted into Opti-MEM I Reduced-Serum Medium (Opti-MEM) (Invitrogen, Aust.) to yield final concentrations of 20, 50, 100 and 200nM in a total volume of 12.5μl. A range of 0.15-0.4μl of Lipofectamine Transfection Reagent (Invitrogen, Aust.) was recommended by

the manufacturer for optimisation, therefore 0.15, 0.3 and 0.4μl, was diluted into 12.5μl of Opti-MEM. These concentrations will be referred to as low, mid and high.

The siRNA oligomer and transfection reagent solutions were then mixed and added to the cell cultures for knockdown experimentation as described in the next section.

8.3.2.3 Transfection procedure

In accordance with the manufacturer's instructions, the transfection reagent and siRNA oligomers were initially prepared as 2 separate solutions. Following the cell culture described above (section 8.3.2.1) each solution was incubated separately for 5 minutes at room temperature before being combined. Once combined the solutions were mixed gently, then incubated for a further 20 minutes at room temperature. During the incubation, each culture was washed twice in Opti-MEM. Following the 20 minute incubation, the combined siRNA and transfection reagent solution was added to each well. Plates were then returned to the incubator at 37°C, 5% CO_2 for 4 hours. Following this incubation, the cells were removed from the incubator and cell culture plates and 75μl of Opti-MEM was added. The cells were then returned to the incubator at 37°C, 5% CO_2 for a further 24 hours.

8.3.2.4 Measuring the efficiency of transfection

Following the 24 hour incubation, the plates were visualised using a Olympus AX 70 fluorescence microscope set to a standard FITC setting (λ excitation = 494 nm, λ emission = 519 nm green). The results from this section provided starting guidelines for the optimal concentrations of transfection and siRNA molecules for efficient knockdown of GPCR43.

8.3.3 Establishing optimal conditions for knockdown of GPCR43

8.3.3.1 Cell culture

Cells from 85% confluent T_{75} flasks were treated with trypsin, counted and $5x10^5$ cells were replated into 10cm tissue culture dishes (Falcon, USA) containing 10ml of media. Dishes were then incubated at 37°C, 5% CO_2 for 24 hours to produce cultures of approximately 30% confluency.

8.3.3.2 Controls

Initially two Stealth RNA oligos (Invitrogen) with 2 different sequences specific to GPCR43, (defined as 43(1) and 43(2)) at concentrations of 100nM and 200nM were tested along with mid and high concentrations of transfection reagent to produce maximal knockdown without toxicity. For each RNAi knockdown, in addition to the oligo designed to knockdown the gene of interest, 3 different controls were required; untransfected, containing media alone, mock transfected containing media and transfection reagent and universal scramble control containing transfection reagent and a siRNA oligomer that does not interfere with any known gene. The controls used in all experiments are described below:

Untransfected control- The untransfected control contains cells which were not exposed to any transfection reagents or siRNA molecules. This control provides the baseline from which to determine if any effects observed in RNAi experiments were due to any component of the transfection process.

Mock Transfected control- The mock transfected control contains cells which were exposed to media and transfection reagent only. This control ensures that any effects observed in RNAi experiments were not due to the transfection reagent alone. Many reports have demonstrated that transfection reagents are highly damaging to the cells (Luo and Saltzman, 2000) (Kiefer et al., 2004) (Huppi et al., 2005). Non-specific changes observed in this control are likely to indicate high levels of toxicity. If the toxicity cannot be overcome with optimisation an alternate transfection reagent may be required.

Scrambled siRNA control- The scrambled siRNA control contains cells which have been exposed to transfection reagent and scrambled siRNA oligos. Scrambled siRNA molecules are siRNA's with sequences that have no homology to any sequence in the human genome. The use of scrambled siRNA molecules should not induce any effect, thereby acting as a negative control (Tschaharganeh et al., 2007). The use of this control allows any results to be evaluated as genuine and due to the siRNA molecule specific to the gene of interest and not the siRNA transfection process.

Positive control- It is also important to investigate the expression of an unrelated gene to again ensure that the knockdown induced was specific to the gene of interest. In the current experiment 18S was investigated. As 18S is a housekeeping gene it can also be

used to ensure that all RNA is evenly transcribed into cDNA as previously described in Chapter 5.

8.3.3.3 Dilutions of transfection reagent

As previously described in section 8.3.2.3, master mixes of each solution were made at the time of experimentation to reduce pipetting error, however the individual volumes have been described. For mid concentrations of transfection reagent, 10μl of Lipofectamine was added to 490μl of Opti-MEM and for high concentrations 15μl of Lipofectamine was added to 485μl of Opti-MEM. The untransfected control did not receive any siRNA or transfection reagent, therefore 500μl of Opti-MEM only was used.

8.3.3.4 Dilutions of siRNA oligos

The scrambled control siRNA oligo and the 43(1) and 43(2) oligo were diluted in RNAse free water to yield a final concentration of 20μM and stored at -20°C. Immediately prior to experimentation, each oligo was diluted to a 2μM working stock and used in experimentation to yield final concentrations of 100nM or 200nM. For 100nM solutions, 25μl of siRNA oligos were added to 475μl of Opti-MEM and for 200nM solutions 50μl of siRNA oligos were added to 450μl of Opti-MEM. Again, master mixes of the solutions were used.

The untransfected and mock control groups did not receive any siRNA exposure, therefore an equal volume of Opti-MEM was added to the cultures at this step.

8.3.3.5 Transfection

As described in section 8.3.2.3, the transfection reagent and the siRNA oligos were initially diluted separately in Opti-MEM prior to being mixed and added to cell culture plates. Plates were then incubated for 4 hours with the siRNA and transfection reagent solution at 37°C, 5% CO_2, before the addition of 9ml of Opti-MEM to the plates and incubation for a further 24 hours at 37°C, 5% CO_2. See section 8.3.2.3 for full details of transfection procedure.

8.3.3.6 Testing the knockdown of GPCR43

After the 24 hour incubation described in the previous section, cells were treated with trypsin and RNA was extracted from the remaining cells according to 2.2.2-2.3.1 and Q-PCR conducted as previously described in 2.4.1. This was to confirm that knockdown of GPCR43 had occurred and to identify which of the conditions tested provided the most significant knockdown.

8.3.4 Testing GPCR43 expression of transfected cells following BuA exposure

In Chapter 7 it was demonstrated that the addition of BuA to HT29 cells induced a down-regulation of GPCR43 receptor expression. Therefore, it was of interest in these experiments to determine if BuA-induced down-regulation of GPCR43 occurred to a similar or greater extent than with RNAi knockdown. Therefore, it was hypothesised that the BuA-induced decrease in GPCR43 expression could be masking any effect of siRNA induced GPCR43 knockdown as the addition of BuA to each of the control groups would reduce GPCR43 to similar levels. In Chapter 7, BuA-induced knockdown was demonstrated conclusively in numerous experiments (3 individual experiments conducted in triplicate across 2 different time points in both high and low glucose media). In the knockdown experiments in this chapter, GPCR43 expression upon the addition of BuA was only examined on one replicate as this response has already been consistently observed.

In the same manner described above, cells from each of the control and GPCR43 knockdown groups were treated with trypsin and used for GPCR43 expression analysis, and for apoptosis and proliferation assays as previously described in 2.6. In addition, $5x10^6$ cells from each treatment group were replating into 6cm dishes in the same manner as 8.3.2.1 and incubated at 37°C, 5% CO_2 for 24 hours. After 24 hours the media was replaced with media containing 0mM BuA or 5mM BuA and incubated at 37°C, 5% CO_2 for 24 or 48 hours. After 24 or 48 hours respectively GPCR43 expression was measured using the methodology described in 2.4.1 to determine if BuA affected GPCR43 expression was altered to a similar or greater extent by BuA or the RNAi process.

8.3.5 Knockdown of GPCR43 and subsequent measurement of BuA-induced changes to proliferation and apoptosis

Once the optimal conditions of GPCR43 knockdown had been identified, it was possible to use those conditions to knockdown GPCR43 in HT29 cells for use in functional

assays, to determine the role of GPCR43 in BuA-induced decreases of proliferation and increases in apoptosis. As the role of glucose in the media was shown to affect both GPCR43 expression and the response of HT29 cells to BuA in Chapter 7, the knockdown cells from the current Chapter were also examined in high and low glucose environments.

8.3.5.1 Cell culture

Cells from 85% confluent T_{75} flasks were treated with trypsin, counted and $5x10^5$ cells were replated into 10cm tissue culture dishes (Falcon, USA) containing 10ml of medium. Culture dishes were then incubated at 37°C, 5% CO_2 for 24 hours to produce approximately 30% confluent cultures.

8.3.5.2 Dilutions of siRNA oligos and transfection reagent

Lipofectamine transfection reagent (10μl) was added to 490μl of Opti-MEM. Twenty-five microlitres of 2μM Stealth RNA oligos specific for GPCR43 (siGPCR43) and 25μl of scramble control were added to 475μl of Opti-MEM. Together the solutions yielded concentrations of 100nM siRNA and mid concentration of transfection reagent. The diluted siRNA and transfection reagent solutions were combined and added to the cell culture as previously described in this Chapter in section 8.3.2.3.

8.3.5.3 Establishing GPCR43 knockdown in cells used in functional assays

Following 24 hours exposure to siRNA, cells were trypsinised and replated into black 96-well plates for measurement of BuA-induced changes in proliferation and apoptosis as previously described in 2.6. RNA was extracted from the remaining cells according to Chapter 2 section 2.2.2-2.3.1 and Q-PCR conducted as previously described in Chapter 2 in section 2.4.1 to measure the amount of GPCR43 knockdown achieved in the cells used in the functional assays.

8.3.6 Statistical analysis

All analyses were conducted using One-Way ANOVAs with Tukey's post hoc testing, and significance was indicated by values $p<0.05$. All statistical analyses were conducted using GraphPad Prism 5 software for Windows.

8.4 Results

For all graphs with multiple treatment groups the following abbreviations have been used, untransfected (untrans), mock transfected (mock), scrambled control (scr) and GPCR43 knockdown (GPCR43).

8.4.1 Testing transfection of HT29 cells

HT29 cells with a starting density of 20,000-25,000 cells using mid and high transfection reagent with 100-200nM BLOCK-iT oligo were shown to fluoresce under a microscope with a FITC filter. Unfortunately, it was not possible to obtain photographs using this equipment, but the results were similar to that seen in Figure 8.2. This experiment indicated that efficient uptake of siRNA molecules was achievable using the transfection reagent Lipofectamine and, therefore, progression to optimising knockdown of GPCR43 was possible.

8.4.2 Optimising knockdown of GPCR43

All combinations of siRNA and transfection reagent gave significant reductions in the expression of GPCR43 at both 24 and 48 hours compared with controls (Figure 8.3 and 8.4). However, after 48 hours less knockdown was observed compared to the 24 hour time point.

The greatest decrease in GPCR43 expression with the lowest concentration of transfection reagent and siRNA molecules was observed with 43(1) with mid concentrations of transfection reagent after 24 hours. Despite higher concentrations of both agents, no further significant decreases in GPCR43 expression were observed, thus 100nM 43(1) and mid concentrations of transfection reagent for 24 hours were used for all future experimentation.

8.4.3 The BuA-induced response of cells with decreased GPCR43 in functional assays

Once the siRNA transfection reagent concentrations had been optimised, knockdown of GPCR43 was conducted in HT29 cells that were then utilised in proliferation and apoptosis assays in both high and low glucose media as described in Chapter 7.

8.4.3.1 Knockdown of GPCR43 in cells used in function assays

The results shown in Figure 8.5 demonstrate that GPCR43 knockdown in the cells to be used in the apoptosis and proliferation assays resulted in approximately 50% less

GPCR43 being expressed compared to untransfected controls. No significant changes in the expression of GPCR43 were detected with mock transfection or scrambled siRNA transfection, which validates the specificity of the knockdown.

To confirm the knockdown of GPCR43 was specific to the gene of interest, the expression of the housekeeping gene 18S was also investigated. Stable expression of 18S indicates that cDNA synthesis occurred evenly across all samples (Figure 8.6).

8.4.3.2 Expression of GPCR43 in HT29 cells with RNAi knockdown in presence of BuA

In the absence of BuA, irrespective of high or low glucose media, the untransfected, mock and scrambled controls expressed similar levels of GPCR43. Significantly less GPCR43 expression is observed with the GPCR43 knockdown group and the controls (Figure 8.7a and 8.7b). However, with the addition of 5mM BuA the mock and scrambled control groups along with the GPCR43 knockdown group showed a significant decrease in GPCR43 expression compared to their respective untreated group. In contrast to the modulation of GPCR43 expression, even expression of 18S occurred in all treatment groups (Figure 8.8a and 8.8b).

8.4.3.3 Proliferative and apoptotic responses of untransfected HT29 cells exposed to BuA

The proliferative and apoptotic response of untransfected HT29 cells to BuA exposure in both high and low glucose media at both time points tested was very similar to the previous results described in Chapter 3 and 7 (Figure 8.9 and 8.10).

8.4.3.4 The effect of GPCR43 knockdown on BuA-induced changes to proliferation and apoptosis

No changes in proliferation or apoptosis were observed with any transfection treatment compared to the untransfected control in the absence of BuA (0mM) at either 24 or 48 hours (Figure 8.9 and 8.10).

There was no significant difference in proliferation between the GPCR43 knockdown group and any of the control groups at the 24 hour time point for cells grown in high and low glucose media (Figure 8.9a). At the 48 hour time point, there were no differences in the proliferative response of cells with GPCR43 knockdown compared with the control

groups except for cells exposed to 20mM BuA when grown in high glucose media (Figure 8.9). At this concentration the BuA-induced decrease in proliferation is reduced in all cells exposed to transfection reagent (mock, scrambled and GPCR43 groups) compared with untransfected controls.

At low concentrations of BuA the knockdown of GPCR43 did not significantly alter the apoptotic response of HT29 cells compared to any control at both 24 and 48 hours (Figure 8.10). Increased apoptosis was seen in all cells exposed to transfection reagent and high concentrations of BuA (10-20mM) compared with the untransfected control, irrespective of whether the cells were grown in high or low glucose media. At the 24 hour time point, this observation was only significant for cells grown in the low glucose media exposed to 10-20mM BuA. At lower BuA concentrations of 5mM-7.5mM any cells exposed to transfection reagent and siRNA molecules (scrambled and GPCR43) showed significantly more apoptosis compared with the untransfected control.

Following the 48 hour incubation, any group which had exposure to transfection reagent and a high BuA concentration demonstrated greater apoptosis than the untransfected cells. This observation was significant at 15-20mM BuA in cells grown in high glucose media.

8.5 Discussion

8.5.1 Finding a suitable RNAi transfection protocol

Many unsuccessful attempts to induce RNAi knockdown for GPCR43 were made using different siRNA molecules, transfection reagents and transfection techniques. However, in this type of experimentation it is difficult to determine which of the steps is failing. Therefore, the use of a fluorescently labelled siRNA molecule was used to determine whether the siRNA molecules were being efficiently transfected into the cells. BLOCK-iT has been used successfully in other studies (Zhou et al., 2005) (Hough et al., 2006) and was also utilised in the current study. The use of the fluorescent oligo enabled siRNA cell transfection to be optimised as the efficient uptake of the oligo leads to “glowing cells” when visualised using fluorescence microscopy. As previously described, no photos were able to be taken with the florescence microscope in the current study, however clear uptake of siRNA molecules could be observed in the mid and high transfection reagents concentrations and siRNA concentrations of 100nM and 200nM (see Figure 8.2 for representational results). This method was a significant

stepping stone in the RNAi transfection procedure, as efficient transfection could be optimised prior to the use of specific siRNAs to knockdown GPCR43. With successful uptake of the siRNA molecules demonstrated, it was possible to use the optimised conditions to transfect cells with siRNAs specific to GPCR43.

8.5.2 Optimal concentrations of transfection reagent and siRNA molecules for GPCR43 knockdown

Several reports (Jackson et al., 2003) (Semizarov et al., 2003) show that high concentrations of transfection reagents and siRNA molecules can cause non specific knockdown. Therefore, it was vital to determine the optimal siRNA and transfection reagent concentration to induce maximal knockdown without inducing non-specific effects. Since strong fluorescence was observed with 100nM and 200nM BLOCK-iT using mid to high concentrations of transfection reagent, these concentrations were used when optimising GPCR43 knockdown.

8.5.2.1 Reduction of GPCR43 expression was greatest after 24 hours

The results from this section show that all tested concentrations of siRNA and transfection reagent induced knockdown at the 24 hour time point (Figure 8.3). At the 48 hour time point (Figure 8.4) there were also significant decreases in the expression of GPCR43 although the magnitude of the decrease was lower than that observed at 24 hours. RNAi knockdown is transient as shown by Vickers et al (Vickers et al., 2003). This group examined siRNA induced knockdown of Bcl-X expression between 8-144 hours. The greatest knockdown was measured at 24 hours with expression returning towards normal levels between 48-96 hours. By 120 hours expression had been restored to normal. Consequently, in the current study cells were exposed to transfection reagent for 24 hours in all future experimentation.

8.5.2.2 Greatest reduction of GPCR43 expression is achieved with 100nM siRNA and the mid transfection reagent concentration

Persengiev et al (Persengiev et al., 2004) established the importance of optimising the siRNA concentration by demonstrating that non-specific effects of siRNA molecules on tissue culture cells were concentration dependent. In the current study, all combinations of transfection reagent and siRNA concentrations induced down-regulation of GPCR43 indicating that any combination was suitable. However, as previously described, exposure to transfection reagents and siRNA can cause non-specific responses and in

some cases the induction of apoptosis, therefore it is vital to use the lowest concentrations of all reagents possible. The results from this Chapter indicate that 100nM of the siRNA denoted GPCR43(1) used with a mid concentration of transfection reagent induced a significant decrease in GPCR43 expression with the lowest concentrations of all other reagents. This combination was, therefore, utilised in subsequent experiments.

8.5.2.3 Control experiments confirmed GPCR43 knockdown was specific to the gene of interest

To ensure knockdown is specific to the gene being investigated it is vital that adequate controls are included to validate experiments (Lehner et al., 2004). In the current experiment GPCR43 expression in the knockdown group was significantly different to each control (untransfected, mock and scrambled). Furthermore, the mock and scrambled controls did not reduce the expression of GPCR43 compared to the untransfected control, validating the experiment and demonstrating that the expression of GPCR43 was specifically reduced by the siRNA targeting the receptor and not the transfection procedure.

8.5.3 Knockdown of GPCR43 in HT29 cells used in functional assays

Following optimisation of GPCR43 knockdown, the optimised conditions were used to knockdown GPCR43 expression in HT29 cells for use in proliferative and apoptotic cellular assays.

8.5.3.1 GPCR43 expression was reduced by approximately 50% using RNAi

The amount of knockdown of gene expression achieved using RNAi depends greatly on the gene of interest, the cell line used and the transfection reagent and process utilised. In the case of GPCR43, other studies have shown successful knockdown of the receptor, however, the extent of the knockdown achieved in each study varied greatly. Yonezawa et al (Yonezawa et al., 2006) demonstrated vastly different knockdown while using identical conditions except for the sequence of the siRNA molecule. This group used 3 different siRNA molecules and showed 30.9%, 88.2% and 99.8% knockdown relative to the scrambled control in the breast cancer cell line MCF-7. Using the siRNA molecule that showed almost complete GPCR43 knockdown of 99.8% in MCF-7 cells, these researchers demonstrated that GPCR43 knockdown cells had decreased cytosolic Ca^{++} release following propionate stimulation and increased phosphorylation of

p38 following propionate stimulation compared to cells treated with scrambled siRNA molecules.

In another study, Hong et al (Hong et al., 2005) achieved approximately 60% knockdown and demonstrated a role for GPCR43 in adipocyte differentiation. The knockdown achieved in the current study was approximately 50% of untransfected control and was significantly different from all control groups (untransfected, mock, scrambled) (Figure 8.5). In the current study many unsuccessful attempts were made to induce greater knockdown of GPCR43 using alternative products, including 3 different siRNA molecules and 2 different transfection reagents. Furthermore another technique known as “reverse transfection” was attempted using Ambion products. The technique involves transfection of the cells with transfection reagent and siRNA molecules as the cells are being plated, rather than 24 hours later once the cells are attached. This technique was demonstrated by Ambion (tech notes # 12(1)) to improve the likelihood of RNAi knockdown in difficult to transfect cell lines. However, in the current study, this did not induce any significant knockdown of GPCR43. The knockdown data arising from experiments using alternate reagents and transfection techniques have not been presented as significant knockdown in GPCR43 expression could not be detected. However, since Hong et al (Hong et al., 2005) demonstrated that 60% knockdown was sufficient to inhibit adipocyte differentiation, functional assays were attempted with 50% GPCR43 knockdown in the current study.

8.5.3.2 Knockdown of GPCR43 using RNAi was specific to the gene of interest

As discussed, it is important to compare knockdown of the gene of interest with mock and scrambled controls, however the transfection process can also affect the expression of other non-related genes. Persengiev et al (Persengiev et al., 2004) used gene arrays to demonstrate that the expression of 1154 genes related to various cellular functions and was non-specifically altered following siRNA treatment. In this study, 18S was used to confirm consistent cDNA synthesis in all samples. In the current study 18S displayed even expression across all samples (Figure 8.6), indicating that siRNA did not non-specifically alter gene expression and cDNA was evenly transcribed from RNA.

8.5.4 Functional assays using HT29 cells with GPCR43 knockdown

Gene expression analysis from section 8.5.3 confirmed that the knockdown of GPCR43 was specific in the HT29 cells, and therefore validated the results obtained from the

experiments investigating the role of GPCR43 in BuA-induced decreases in proliferation and increases in apoptosis.

8.5.4.1 Changes to the proliferative and apoptotic response of HT29 cells following BuA exposure occur as expected

BuA-induced changes to the proliferation and apoptosis of untransfected cells in the current Chapter occurred in a comparable manner to results obtained for similar experiments in previous Chapters. A discussion of these results can be found in Chapter 3 and 7. The results from the current Chapter also indicate that the experimental procedure required to conduct RNAi transfection investigations does not alter the ability of HT29 cells to respond to BuA.

8.5.4.2 The transfection procedure did not alter the proliferative or apoptotic response of cells in the absence of BuA

No differences in the proliferation or apoptosis between any treatment groups (untransfected, mock, scrambled, GPCR43) were observed in the absence of BuA. This indicates that the concentrations of siRNA and transfection reagents are adequate to induce knockdown, but low enough to prevent non-specific alterations in proliferation (Figure 8.9 and 8.10).

8.5.4.3 GPCR43 does not appear to regulate BuA-induced decreases in proliferation

After 24 hours and 48 hours the only significant difference in proliferation between the untransfected HT29 cells and the cells in all other treatment groups was seen at 24 hours following exposure to 20mM BuA in high glucose media (Figure 8.9). At this concentration a reduction in the BuA-induced decrease in proliferation was observed. However, as this effect is seen with the addition BuA in all groups exposed to transfection reagent (mock, scrambled, GPCR43) it is likely that this observation is a non-specific effect arising from exposure to transfection reagent. Therefore, the results from this section indicate that the BuA-induced changes to proliferation observed in this Chapter are not due to specific GPCR43 knockdown.

8.5.4.4 GPCR43 does not appear to regulate BuA-induced increases in apoptosis

At low concentrations of BuA the knockdown of GPCR43 did not alter the apoptotic response of HT29 cells following BuA exposure compared to controls at either time point examined, irrespective of whether cells were grown in high or low glucose (Figure 8.10). This indicates that GPCR43 is not likely to modulate BuA-induced apoptosis. However, non-specific effects of the transfection process were observed following exposure to mid and high BuA concentrations. Greater increases in apoptosis were seen in the treatment groups exposed to transfection reagent compared with the respective untransfected control following mid to high concentrations of BuA treatment (Figure 8.10). Tschaharganeh et al (Tschaharganeh et al., 2007) observed similar results and stated that "because most studies focus on verification of expression profiles after siRNA transfection only limited data regarding the potential functional consequences are currently available". They (Tschaharganeh et al., 2007) demonstrated that 2 commonly used scrambled siRNA molecules were sufficient to alter cell viability, proliferation, cell cycle, migration and apoptosis compared with untransfected controls. In this study, transfection reagent alone did not significantly alter cell proliferation or apoptosis, however with the added cellular stresses induced by BuA treatment the results did show that transfection reagent contributed to the magnitude of these effects. In the current experimentation the concentrations of transfection reagents and siRNA molecules were optimised to reduce non-specific knockdown. However, with the addition of BuA, non-specific effects of the transfection reagents and siRNAs were still observed. This indicates that a new technique without the effect of these non-specific responses may be required.

8.5.5 BuA induces greater knockdown of GPCR43 than siRNA

The results from this Chapter also demonstrate that the reduction of GPCR43 expression induced by 5mM BuA was greater than that achieved using RNAi (Figure 8.7). This poses a significant problem as BuA must be included in these experiments to produce BuA-induced effects, but as BuA itself modulates GPCR43 expression it is difficult to ascertain the role of this receptor in cellular activities. In light of this observation, it appears that investigation into BuA-induced changes to apoptosis and proliferation could not be conducted accurately using the RNAi knockdown technique, unless the knockdown could be further reduced to a level similar to Yonezawa et al (Yonezawa et al., 2006) with 99.8% knockdown. If this occurred the mechanisms of action of BuA that occur through GPCR43 could be determined, as with virtually no receptor present BuA would no longer be able to elicit its effects on these cells. If no

change in response was observed, one could more confidently state that GPCR43 was not involved in the proliferative and apoptotic effects of BuA.

8.5.6 GPCR43 knockdown in BuA-induced changes to apoptosis and proliferation: conclusions

The non-specific effects induced in the current study only occurred in the groups exposed to transfection reagent following the addition of mid to high BuA concentrations. It is likely therefore, that the combination of damage induced by transfection reagent and siRNA molecules coupled with exposure to BuA was sufficient to induce higher levels of apoptosis in these groups. Despite the non-specific effects induced at some concentrations of BuA, in most instances there were no significant differences between any treatment group and GPCR43 knockdown for either proliferation or apoptosis. The results from this Chapter suggest that GPCR43 is not involved in a rate limiting manner in BuA-induced changes in apoptosis and proliferation in HT29 cells.

It is possible that the 50% knockdown achieved was not adequate to reduce functionality of the receptor. However, Hong et al showed 60% knockdown of GPCR43 was sufficient to affect differentiation; therefore the 50% knockdown achieved in the current study was pursued in proliferation and apoptosis functional assays. As described earlier in this Chapter, a number of alternative siRNA molecules, transfection reagents and transfection techniques were used in an attempt to induce knockdown greater than 50%. Future experimentation could utilise the siRNA protocol published by Yonezawa et al (Yonezawa et al., 2006) which yielded 99.8% knockdown of GPCR43. However, it is difficult to replicate exact results using siRNA in different cell lines as each responds differently and some can be more difficult to transfect. Yonezawa et al (Yonezawa et al., 2006) used MCF-7 cells, so even if the exact protocol was replicated it may not yield the same level of knockdown in HT29 cells.

To overcome the issues raised in this section, it would be vital to reduce the level of non-specific effects induced by transfection reagents or find an alternative method for investigation of GPCR43 function.

8.5.7 Overcoming the non-specific effects of RNAi transfections following BuA exposure

Despite the non-specific effects only being present with BuA addition, in future experiments it would be important to overcome these issues. Problems with non-

specific effects related to siRNA are not unique to the current study, with other groups recognising that this issue is the “current challenge associated with this technique” (Jackson and Linsley, 2004). The current study, however, is encouraging as non-specific effects are only present with the addition of BuA so further developments in transfection reagents and other components of siRNA transfections may reduce these effects even further. Trialling a number of different transfection reagents may be fruitful in attempting to reduce toxicity, but as BuA will also be required in these assays almost complete knockdown on the receptor will be required. Since BuA is an apoptosis inducing agent any cells exposed to the toxic effects of a transfection reagent will be likely to undergo greater BuA-induced apoptosis compared with untransfected controls. It may be possible to use electroporation to transfect the cells, however in many instances this technique has also been shown to induce significant cell damage (Huppi et al., 2005) including both necrosis and apoptosis (Pinero et al., 1997). Another possible way to overcome this issue would be to switch to a GPCR43 knockout mouse model; as cells from these animals would have no expression of GPCR43 and would not have been exposed to any reagents that increase cell apoptosis. However, with the mouse knockout model it is possible that this methodology will have other considerations that limit definitive experimentation. Dass et al (Dass et al., 2007) hypothesised that GPCR43 was involved in cell motility and utilised a GPCR43 knockout mouse model in their experiments. Although 2 other research groups had published results indicating a role for GPCR43 in intestinal motility, the mouse model used by Dass et al failed to show any function of GPCR43 in intestinal motility. This group describes the results as “initially surprising”, then suggested that it remained a “possibility that the function of the GPR43 receptor has been conserved within the knockout mouse by up-regulation of another receptor, such as GPCR41”. Therefore, knockout animal models may not be the solution to the difficulties experienced in this chapter, and it may be some time before new techniques are developed to produce the results required to definitively answer the questions posed in this Chapter.

8.5.8 Down-regulation of GPCR43 may mediate BuA-induced effects of this receptor

BuA causes a down-regulation of GPCR43, and therefore it may be that the down-regulation of the GPCR43 receptor mediates BuA effects downstream. Therefore, use of a knockout model may enhance the effect of BuA and not provide any information about the function of this receptor. This could be investigated by transfection of the

GPCR43 receptor into cells to induce over-expression with subsequent measurements of BuA-induced changes to apoptosis and proliferation.

8.5.9 Final summary and conclusion

The results of the experiments described in this Chapter suggest that GPCR43 does not appear to be involved with BuA-induced changes to apoptosis or proliferation. However, this conclusion must be interpreted with caution as (1) there may not have been sufficient knockdown to effect a change and (2) the addition of BuA reduces the level of GPCR43 expression to similar levels in RNAi control and treatment groups. Furthermore, other groups have suggested that the knockdown of GPCR43 may evoke an up-regulation of the closely related GPCR41 receptor to preserve the function of GPCR43. Therefore, an alternative to determine whether GPCR43 is involved in BuA-induced changes to apoptosis and proliferation would be to use a GPCR43 knockout mouse to investigate the function of this receptor. However, as described this model may bring its own limitations and challenges and if no significant changes were observed in this model substantial experimentation would be required. Finally, the transfection of GPCR43 into cells to induce over-expression may be a useful tool for investigating GPCR43 function in the future.

9 Expression of GPCR43 in patient samples

9.1 Introduction

In Chapters 6 and 7 interesting observations have demonstrated that GPCR43 is differentially expressed in different cellular conditions; including cell confluence, exposure to its agonist BuA and altered glucose concentration. Furthermore, in Chapter 5 it was demonstrated that although 6 different CRC cell lines possessed a copy of the GPCR43 gene, the expression of the receptor differed between the cell lines, with 2 indicating no GPCR43 expression. The highly diverse expression of GPCR43 in these situations prompted interest into the expression profile of human patient samples and in particular, any differences between normal and tumour samples.

Obtaining human colon samples was a high priority in the current project, and this was made possible late in the project through a collaboration with Dr Andrew Ruszkiewicz, Senior Medical Pathologist, Institute of Medical and Veterinary Science (IMVS), Adelaide South Australia. A pilot study utilising 8 normal and 11 tumour samples was conducted using tissue obtained from surgical biopsies that had been processed, snap frozen in liquid N_2 and stored at -80°C. As molecular changes are the underlying basis of cancer, the measurement of these changes can provide useful information to link genetic changes to pathological observations (Goldsworthy et al., 1999) and potentially provide information relating to the mechanisms involved in cancer development and progression. Investigation of the expression of GPCR43 in normal and cancerous colon samples may also provide insight into the role of this receptor.

Although the genetic analysis of normal and tumour tissue can be useful for the identification of genes that are differentially expressed in disease and normal tissue (Upson et al., 2004), it has now been recognised that the tissue microenvironment (Guda et al., 2003) (Birkenkamp-Demtroder et al., 2002) (Lin et al., 2002) and the tissue surrounding the tumour can play a role in cancer development and progression (Sugiyama et al., 2005). In the case of CRC, the stroma surrounding the cancerous epithelium plays an important role in cancer growth, invasion and metastasis (Bhowmick et al., 2004) (Calvert and Frucht, 2002; Chung, 2000) (De Wever and Mareel, 2003), with Sugiyama et al (Sugiyama et al., 2005) demonstrating differential patterns of gene expression in cancer cells and surrounding stroma compared with their normal counterparts. Other factors also affect the tissue surrounding tumours, with a number of groups demonstrating lymphocyte infiltration into many solid tumours such as breast

(Aaltomaa et al., 1992), lung (Furukawa et al., 1985), melanoma (Clemente et al., 1996) and colorectal cancers (Ropponen et al., 1997) (Jass, 1986) (Svennevig et al., 1984).

When conducting a genetic analysis of normal compared with cancerous samples it is possible that substantial lymphocyte infiltration into the stroma of tumour samples may be sufficient to alter the expression levels within the tumour, especially if the gene of interest is expressed in lymphocytes. GPCR43 has been shown to be highly expressed on cells of the immune system, including lymphocytes (Brown et al., 2003) (Brown et al., 2005) (Le Poul et al., 2003). Therefore, the possibility of lymphocyte infiltration altering GPCR43 expression in CRC tumour samples compared with normal was investigated using laser capture microdissection (LCMD) technology. LCMD allows the extraction of pure cell populations from heterogenous samples such as tissue biopsies (Upson et al., 2004) (Zhuang et al., 1995) (Shibata et al., 1992) (Whetsell et al., 1992) and the subsequent genetic analysis of homogenous cell populations. In the current study LCDM was used to isolate epithelial cells from the stroma of CRC patient samples.

9.2 Approach

To determine the level of GPCR43 expression on normal and tumour patient biopsies using whole tissue samples or samples processed by LCDM.

9.3 Methods

9.3.1 Ethics

Ethical approval for the work conducted in this experimentation was obtained from the IMVS and CSIRO human ethics committee.

9.3.2 Collection and staging of human samples

All samples were kindly supplied by Dr Andrew Ruszkiewicz who also provided information about the staging of disease using Duke's staging methodology as seen in Figure 1.2 in Chapter 1. Samples were collected from patients who had undergone surgery to remove cancerous lesions and snap frozen in liquid N_2. Normal samples were collected from patients with normal mucosa or are samples of normal tissue taken from patients with cancer. Table 9.1 shows the staging for each patient sample. In the case of normal samples taken from patients with cancer the stage of the disease has been recorded in this table.

9.3.3 Isolation of total RNA from human CRC patient tissue samples

Due to the small amount of tissue in each sample, RNA was extracted from human patient samples using Trizol Reagent (Invitrogen, Aust.) instead of the spin column format described previously in Chapter 2. Tissues from human patient samples were cut into sections 20μM thick using a Microm Microtome Cryostat HM 525 at -20°C and collected into 1.5ml tubes. Trizol Reagent (1ml) was added to the tubes, and samples were homogenised by passing through a 20 gauge needle 20 times. Total RNA was then extracted according to the Trizol manufacturer's instructions. Samples were stored at -80°C until used in further experimentation.

RNA quality and quantity was investigated using the NanoDrop system as described previously in Chapter 2.

9.3.4 Additional gDNA digest of RNA from human CRC patient tissue samples

In a similar way to the RNA extraction using spin columns described in Chapter 2, there was a need to remove any contaminating gDNA that may have remained in the RNA samples obtained from the Trizol extraction of the samples used in this Chapter. DNA digestion was conducted using the DNA-*Free*™ kit (Ambion, USA #AM1906) to maximise the yield of RNA following the DNase treatment. All procedures were conducted according to the manufacturer's instructions.

9.3.5 Expression of GPCR43 on human patient samples

RNA extracted from human samples was reverse transcribed to cDNA using the methodology described in 2.2.2-2.3.1, and Q-PCR reactions were conducted in triplicate using the methods described in 2.4.1.

The Q-PCR assay was validated by assessing 18S expression in triplicate as described in section 5.3.7.

9.3.6 Fixing and staining of tissue samples for Laser capture

A subset of patient tumour samples was randomly chosen for laser capture experiments to allow the analysis of homogeneous cell types from a heterogeneous cell population. Tissue was cut into 20μM sections using a Microm Microtome Cryostat HM 525 at -20°C and placed onto slides. Slides were then placed into 100% ethanol (stored at -20°C) for 5 minutes and subsequently rinsed in deionised water. This was followed by a

Haemotoxylin stain for 30 seconds, rinse in deionised water, differentiation in acid alcohol for 30 seconds and a further rinse in deionised water. Sections were then placed in Scott's Reagent for 30 seconds before another rinse in deionised water. Finally the sections were submerged in 100% ethanol for 30 seconds before being transferred to a fresh 100% ethanol solution where they remained for 2 minutes. Slides were then air-dried and used for laser capture experiments.

9.3.7 Laser capture of tissue samples

The colonic epithelium was isolated from all other cell types, such as stroma and blood borne cells, in the human patient biopsies. Slides, prepared in 9.3.5, were loaded onto the stage of a P.A.L.M. Microbeam Laser-System for Micromanipulation with UV-A Laser microscope. The RNA preservative, RNAlater® (Qiagen, Aust.), (80μl) was pipetted into the lid of a 0.5ml tube and placed upside down into the movable arm located on the microscope. The arm was then moved so that the tube cap was located directly over the tissue section on the slide, as shown in a pictorial representation of LCMD, adapted from Pinzani et al (Pinzani et al., 2006), in Figure 9.1 a, b and c. The microscope image was observed using computer P.A.L.M RoboSoftware, version 2.2-0103 software and guide lines for the laser were traced around the colonic epithelium, shown in green in Figure 9.2. This instructed the laser to cut the area inside the lines, designated blue in Figure 9.2. As the laser cut the tissue section, small fragments of the tissue were catapulted into the cap of the 0.5ml tube containing RNAlater as shown in Figure 9.1. Samples were then stored at -20°C prior to RNA extraction as discussed in section 2.2.2. Once extracted the RNA samples were stored at -80°C.

9.3.8 RNA quality using Bioanalyzer 2100

Due to the small amounts of sample obtained, it was not feasible to check RNA quality on an agarose gel as described in Chapter 4. Furthermore, collaborators at CSIRO Livestock Industries (CSIRO LI) wished to utilise the RNA to conduct their own genetic analysis. Consequently the RNA samples were couriered on dry ice to CSIRO LI. Here the quality of the RNA samples were analysed using a Bioanalyzer 2100 (Agilent Technologies) according to the manufacturer's instructions. The Bioanalyzer 2100 operates on the principle of electrophoretic separation on microfabricated chips. The RNA samples are separated and the fluorescence of each sample detected by lasers. This has the advantage of generating a more comprehensive RNA analysis whilst only using a very small volume of RNA sample (1μl). An electropherogram is generated by the Bioanalyzer 2100 as well as an image highly similar to an agarose gel. The

electropherogram provides visual analysis of the quality of RNA (Schroeder et al., 2006). A representational diagram of an electropherogram of high quality RNA can be seen in Figure 9.3.

9.4 Results

9.4.1 RNA extracted from whole tissue

Table 9.2 shows the RNA quality and quantity of the 8 normal and 11 tumour patient samples. All normal samples had A_{260}/A_{280} values of >1.8 indicating RNA of high quality. Samples 1 and 4 from the 11 tumour samples did not register any absorbance value when using the NanoDrop so these samples were excluded from further analysis. The starting amount of tissue from the tumour biopsies 1 and 4 were much smaller than the other tumour samples, with only 1 section being cut using the cryostat. The lack of sample is, therefore, likely to be responsible for the absence of any detectable RNA.

9.4.2 Expression of GPCR43 in whole tissue

Figure 9.4 shows the expression of GPCR43 on 9 normal biopsies and 9 tumour biopsies which had not been laser dissected. For the 9 normal samples the average expression of GPCR43 was 768 ± 84 compared to the average expression of the tumour samples 4909 ± 903. There was a much greater variation in the expression of GPCR43 in the tumour samples, with samples 2, 9, 10 and 11 giving expression values similar to those observed with the normal biopsies. The staging of the disease conducted by Dr. Andrew Ruszkiewicz using the Duke's methodology did not show any correlation between GPCR43 expression and severity of disease as observed in Figure 9.5.

18S expression was consistent across all normal and tumour samples except for sample normal 8 which was elevated (Figure 9.6).

9.4.3 Laser dissected RNA

Four of the 11 tumour samples were randomly chosen for LCMD. Table 9.3 shows the RNA quantity and quality of the samples. It can be observed none of the RNA samples from these extractions after LCMD passed the criterion of $A_{260/280}$ ratios values >1.8. Additionally, Table 9.3 shows multiple negative values for $A_{260/280}$ and concentration. These results produced a negative concentration of RNA in one sample and a negative $A_{260/280}$ value for two other samples, indicating that the quality of these samples was compromised. One sample (CRC3) did not have negative values, however, purity was

1.37 which was too low to be classed as acceptable. The quality of these samples was not adequate for use in Q-PCR experiments when tested using the NanoDrop and therefore were not sent to CSIRO LI for further analysis using the Bioanalyzer 2100.

9.4.4 RNA quality assessed using Bioanalyzer 2100

9.4.4.1 Normal human colon samples

Human tissue samples were examined along a positive control containing high quality RNA from rat colon tissue extracted by Mr Barney Hines, CSIRO LI, using Trizol. Distinct peaks at approximately 42 and 50 seconds can be seen on the electropherogram of this sample (defined as Rat Trizol) in Figure 9.7. Strong 18S and 28S bands can also be seen in Figure 9.8, lane 9, for this sample. The results from the 8 normal human tissue samples showed that degradation of the RNA had occurred as no samples contained strong peaks at approximately 42 or 50 seconds on the electropherogram (Figure 9.7) or 18S and 28S rRNA bands (Figure 9.8).

9.4.4.2 Colon cancer samples

In the tumour samples the same RNA sample extracted by Mr Barney Hines with known RNA integrity was used as a positive control (defined Rat Trizol). The RNA extracted from the cancerous human samples were degraded in most instances with only sample 8 showing distinct peaks at 42 and 50 seconds on the electropherogram (Figure 9.9) and strong 18S and 28S expression (Figure 9.10). In Figure 9.10 faint 18S and 28S bands can be observed in samples 3, 5, 6 and 7.

9.5 Discussion

9.5.1 RNA extracted from whole tissues

When quantitatively investigating gene expression, RNA degradation can lead to highly inaccurate results as the data obtained may reflect fluctuations in the degradation of the message rather than the true changes in expression (Bustin, 2002; Bustin and Nolan, 2004a) (Fleige and Pfaffl, 2006; Fleige et al., 2006). The extraction of RNA from whole tissue samples was conducted successfully and all samples showed good quality when tested using the $A_{260/280}$ measured with the NanoDrop. Although this technique is not as comprehensive as more recent technologies such as the Bioanalyzer 2100 (which will be discussed later in this Chapter) the absorbance values were all greater than 1.8 (Table 9.2) which is considered a quality cut off (Fleige and Pfaffl, 2006). The

absorbance is measured across wavelengths 220-750nm creating a “profile” or peak with the highest point at 260nm and the lowest at 280nm. As well as $A_{260/280}$ values greater than 1.8 all samples tested in the current experiment showed routine nucleic acid profiles when tested using the NanoDrop.

9.5.2 GPCR43 expression in whole tissue samples

In this initial pilot study investigating the expression of GPCR43 in normal and cancerous colon biopsies there appears to be increased GPCR43 expression in tumour compared to normal samples. In whole tissue samples, the average expression of GPCR43 is 768 ± 84 for normal biopsies and 4909 ± 903 for tumour biopsies. It must be noted however in this pilot study that the tissue samples are not normal and tumour matched from the same patient, nor are they age or sex matched. These factors are vital when assessing the differential expression of any gene, and must be addressed in larger studies of this type. The staging of the disease, using Duke’s methodology, was assessed in the samples used in this study and did not show any correlation to the expression of GPCR43 (Figure 9.5). This is not, however, surprising as any correlation would be difficult to ascertain with the small sample group that was used in this study.

Although there is a large variation in the expression of GPCR43 in the tumour samples (Figure 9.4), this result is not unlike the result from Chapter 4 and 5 which investigated the expression of GPCR43 in 6 different CRC cell lines. In these Chapters, there was a significant variation in GPCR43 expression across the cell lines, with some cell lines not expressing the receptor (Chapter 4 and Chapter 5) despite possessing a copy of the gene (Chapter 4 Figure 4.6). This is an intriguing observation as it may indicate that transcription of this gene rather than translation or post-translational modifications is important in the changes in receptor expression during cancer development and this effect is highly variable.

9.5.3 Laser captured RNA

It is thought that lymphocyte infiltration into the tumour site is an immunological response (Clemente et al., 1996) and it has been well documented that the survival outcomes of patients with CRC is positively correlated with higher lymphocyte infiltration into the stroma of the tumour (Jass, 1986) (Ropponen et al., 1997) (Takemoto et al., 2004). As discussed in the introduction, lymphocyte infiltration into solid tumours has been described by a number of research groups. The possible effect of lymphocyte infiltration on GPCR43 expression was of great interest in this project as Brown et al in

2003 and 2005 (Brown et al., 2003) (Brown et al., 2005) and Le Poul et al (Le Poul et al., 2003) demonstrated GPCR43 to be most highly expressed in immune cells such as monocytes (Brown et al., 2003) (Le Poul et al., 2003), neutrophils (Brown et al., 2003) and PBMCs (Brown et al., 2003) (Le Poul et al., 2003) (Brown et al., 2005).

To elucidate whether the increased expression of GPCR43 in tumour samples was due to lymphocyte infiltration into the stroma, LCMD of the colonic epithelium from 4 tumour samples was conducted. It was intended that the GPCR43 expression results from the 4 laser dissected samples would be compared with the matching tumour samples to determine if lymphocyte infiltration altered GPCR43 expression. Results in Table 9.3 demonstrate that the quantity of the RNA extracted was between 14.32ng/μl to 91.94ng/μl. However, no detectable quantity of RNA was obtained from the 2 other samples. It was previously ascertained in Chapter 5 that the minimum RNA quantity required for RT reactions to detect GPCR43 in subsequent Q-PCR assays was 1μg (see Figure 5.7 and section 5.5.1.5). Although the experiment in Chapter 5 was conducted using RNA extracted from HT29 cells, the experiment provides a good guide for determining the minimum RNA quantity required. The low quantities of RNA extracted in the current Chapter made it impossible to add 1μg of RNA to the cDNA synthesis reactions without exceeding the total reaction volume of 25μl. Consequently it was not feasible continue to process these samples. The results obtained in the current study are not unlike other studies which have also produced small quantities of RNA from LCMD. Using LCMD, the total starting amount of RNA for cDNA synthesis or RNA amplification reactions in other studies were 10ng (Luzzi et al., 2001), 37.5ng (Upson et al., 2004), and 100ng (Alevizos et al., 2001). In an assessment of different staining protocols prior to LCMD, Morrogh et al (Morrogh et al., 2007) demonstrated RNA concentration and quality ranged between 0.38ng/μl-49.7ng/μl and $Ab_{260/280}$ values of 1.5-2.54. Additionally, Luzzi et al (Luzzi et al., 2001) stated that in the majority of reports, the RNA quantity resulting from LCMD was insufficient for nucleic array based assays.

In addition to the low yields of RNA, another issue which must be considered is RNA quality. The quality of the LCMD samples obtained in this study was not acceptable for use in Q-PCR experimentation as evident by the low $Ab_{260/280}$ values. The issue of low quality RNA following LCMD has been reported by many other groups. The quality and quantity of mRNA isolated from LCMD can be affected by tissue collection, fixation technique (Goldsworthy et al., 1999), sectioning, storage of the samples (Goldsworthy et

al., 1999) (Fend et al., 1999) (Kohda et al., 2000) (Morrogh et al., 2007) and by the tissue processing during LCMD itself (Vincent et al., 2002). Furthermore, Foss et al (Foss et al., 1994) suggested that the prolonged exposure times required for fixation procedures may increase RNA degradation due to increased exposure to endogenous RNAses.

Unfortunately in this study sufficient quantities of RNA with suitable quality were not extracted using LCMD. This issue could be potentially overcome with significant optimisation of the technique, but as the quality and quantity of the RNA is linked to multiple parameters as described above, this was beyond the scope of this project.

9.5.4 Is the increased GPCR43 expression in CRC samples related to lymphocyte infiltration?

Despite the possibility that lymphocyte infiltration may affect GPCR43 expression in CRC samples, Jass et al (Jass, 1986) demonstrated that there was a negative association with lymphocyte infiltration and the progression of disease indicated by Duke's staging. This group measured 447 rectal cancer biopsies and found 53% of stage A cancers, 28% of stage B cancers and 13% of stage C cancers showed substantial lymphocyte infiltration. A similar result was demonstrated by Ropponen et al (Ropponen et al., 1997) who demonstrated a linear association of lymphocyte infiltration with Dukes staging, and noted that in large invasive Dukes stage C or D tumours lymphocytes were weak or absent. Despite increased expression of GPCR43 in tumour samples, no relationship between GPCR43 expression and Duke's staging was observed in this pilot study. This indicates that the expression of GPCR43 is not likely to have been induced by lymphocyte infiltration as this has a linear relationship with Duke's staging of disease. Additionally, even with dense lymphocyte infiltration, which was defined by Ropponen et al (Ropponen et al., 1997) as >100 lymphocytes in 1 microscopic field (40X magnification) averaged over 10 fields, the number of lymphocytes compared with epithelial cells may be unlikely to change the total tumour gene expression. However, these hypotheses are not conclusive, and further experimentation is required to show that the increased GPCR43 expression is due to a genuine up-regulation of GPCR43 in colon cancer tumours, and is not just an artefact of lymphocyte infiltration.

9.5.5 Future investigations of GPCR43 expression would require the detection of protein using immunohistochemistry

There has been one study published by Takemoto et al (Takemoto et al., 2004) which casts doubt onto the usefulness of LCMD in investigating lymphocyte infiltration in CRC samples. In this study, antibody staining was used to clearly show lymphocyte infiltration occurs in the stroma and between the epithelium itself. This paper indicates that the laser capture of pure epithelium populations from total tumour biopsies would not be able to determine if the increase in GPCR43 expression in tumour samples was due to lymphocyte infiltration as the lymphocytes could also be present in between the epithelium. Therefore, rather than embark on a large scale study of GPCR43 expression using mRNA, the best recommendation would be to investigate the protein levels of GPCR43 using immunohistochemistry (IHC). This technique allows the localisation of the receptor expression to be visualised. Using IHC, the expression of GPCR43 in the epithelium or in the surrounding stroma could be clearly differentiated. The importance of detecting the GPCR43 protein is recognised and proven by recent publications investigation GPCR43 protein levels (Karaki et al., 2006) (Karaki et al., 2007). Karaki et al created their own antibody specific for rats (Karaki et al., 2006) and humans (Karaki et al., 2007), and using IHC demonstrated the localisation of GPCR43 expression in both rat (Karaki et al., 2006) and human colon (Karaki et al., 2007). In the current study, attempts to detect the GPCR43 protein using commercially available antibodies were conducted. The data has not been presented as all experiments were unsuccessful, however, the future importance of this approach will be described in the final discussion. As the commercially available antibodies were not able to be utilised in the current experiment the development of an antibody to GPCR43, or sourcing one from another research group, would be an important step in ongoing experimentation.

9.5.6 RNA quality tested using Agilent

Equipment such as the the Bioanalyzer 2100 are now considered to be the leading technology for RNA integrity analysis (Fleige and Pfaffl, 2006; Fleige et al., 2006). This type of instrument generates a quantifiable measure of RNA integrity, a quantitation estimate, and will calculate the ratio of the 18S and 28S ribosomal bands all with just 1μl of RNA sample. Unfortunately as the Bioanalyzer 2100 was not available for use in the current study's laboratory the samples were sent to CSIRO LI. Despite all samples showing good $Ab_{260/280}$ values when tested in the current laboratory (Table 9.2), most samples were shown to be degraded when investigated at CSIRO LI using the Bioanalyzer 2100 (Figure 9.7-9.10). Since the Bioanalyzer 2100 gives a more

comprehensive measure of RNA quality it is difficult to directly compare the results of RNA quality in the current laboratory and at CSIRO LI. However, due to the discrepancy in quality before and after shipping it is possible that the samples were degraded during transit to CSIRO LI or during storage prior to or after transportation.

In the results obtained for the tumour samples using the Bioanalyzer 2100, sample 8 had distinct peaks at 42 and 50 seconds on the electropherogram along with strong 18S and 28S rRNA bands (Figure 9.9 and 9.10 respectively) indicating the RNA integrity was intact. Faint but detectable 18S and 28S bands were observed in samples 3, 5, 6 and 7 (Figure 9.10). These sample numbers corresponded to the highest expression of GPCR43 as seen in Figure 9.4. This indicates that the samples with the highest quality RNA had the most expression of GPCR43. Degraded samples are likely to give decreased gene expression values, therefore this result was not considered unusual.

Despite this result, 18S expression was investigated as a loading control and to determine if cDNA synthesis had occurred evenly across all samples. Although these results show more variation in the Ct values of 18S compared with the cell lines tested in other Chapters, the pattern of 18S expression is mostly consistent across all samples, except normal 8 (Figure 9.6). Furthermore, the large fluctuations in the expression of GPCR43 in the tumour samples do not follow the same profile as the small fluctuations in housekeeping gene expression, indicating that changes in overall gene expression were not the cause of the differential GPCR43 expression. As outlined in detail in Chapter 5, housekeeping gene expression has been shown to fluctuate greatly in some conditions, in particular when comparing normal tissue to cancerous tissue. Therefore, with strong and mostly consistent 18S expression in all but 1 sample it is possible that the RNA samples were intact at the time of the GPCR43 genetic analysis and were subsequently degraded.

To overcome this problem, in future experimentation it would be vital to establish standard operating procedure acceptable to all collaborators to minimise the chances of degradation in samples. The samples in the current study had been processed by snap freezing in liquid nitrogen prior to the studies commencement. However, Kihara et al (Kihara et al., 2005) demonstrated that RNA degradation is reduced if stored in a preservative such as RNAlater. Therefore, if levels of GPCR43 mRNA were to be investigated, the use of RNAlater is one example of a modification to the current procedure that could be incorporated into a new standard operating procedure.

9.5.7 Summary and future directions

The pilot study described in this Chapter produced some interesting results, but also highlighted a number of areas where modifications to experimental approach and further optimisation are required. As the samples were only obtained late in this project and limited samples were available, modifications and optimisation were not possible. However, the established collaboration achieved by the current project will allow for the investigation of greater sample numbers and the generation of a tissue bank with matched normal and tumour samples.

9.5.8 Conclusion

Although a very small sample size was utilised and there are clear limitations in this research, an interesting observation that GPCR43 is up-regulated in cancer patient samples was observed which warrants further experimentation. This would be best achieved by the use of suitable GPCR43 antibodies for use in IHC to determine the protein levels of GPCR43 and the localisation of the receptor.

10 Discussion

10.1 Thesis summary

There is strong evidence to suggest that fibre and resistant starch are beneficial for the prevention of CRC. More specifically, BuA, a breakdown product arising from bacterial fermentation of RS, has been demonstrated conclusively to decrease proliferation and induce terminal differentiation and apoptosis in CRC cells *in vitro*. In contrast, BuA acts as the preferential energy source for normal colonocytes *in vivo*. The apparent paradoxical effect of BuA has prompted much interest into this compound for its potential in preventing CRC and as an anticancer therapeutic. Despite a vast array of research and published literature very little is known about the exact biological mechanisms of BuA action. At the commencement of this project a family of GPCRs, GPCR40-43, were shown to be activated by FAs and SCFAs. GPCRs may act like sensors on the surface of cells and play a significant role in a magnitude of physiological and pathophysiological processes. Therefore, these receptors were hypothesised as possible mediators of BuA-induced effects, such as changes to proliferation and apoptosis. Of the GPCR40-43 family, GPCR43 was considered the most likely to be a "BuA" receptor and was investigated in the current project.

The hypothesis examined in this thesis was that GPCR43 acts as an extracellular BuA sensor in the colon to enable colonocytes to sense and adapt to changing nutrient availability and ensure colonic homeostasis by modulating BuA-induced changes to apoptosis and proliferation. This study gained some evidence to support the hypothesis that GPCR43 may be an extracellular BuA sensor, which will be discussed further in this Chapter.

The major aims of this study, as outlined in Chapter 1, can be summarised into 2 broad and overarching aims:

1. Is GPCR43 present on CRC cell lines and human colonic tissue and is the expression modulated by external factors or progression of CRC?
2. Does GPCR43 play a role in one of the functions of BuA i.e. induction of apoptosis and differentiation or the modulation proliferation?

In this Chapter, the results from the current study have been summarised and discussed in relation to these aims and the published literature.

10.2 GPCR43 is expressed in CRC cell lines and human colonic tissue

GPCR43 is most highly expressed on immune type cells (Le Poul et al., 2003) (Brown et al., 2003; Brown et al., 2005) and at the commencement of this project expression of GPCR43 had not been confirmed in colonic tissue. In this project RT-PCR and Q-PCR methods were developed to investigate the expression of GPCR43 in:

1. Six different CRC cell lines derived from adenocarcinoma, carcinoma and metastatic disease.
2. CRC cell lines following changes to cell confluence and nutrient availability to determine if modulation of expression occurred.
3. Biopsies of patients with different stages of CRC and comparison to normal colonic biopsies.

10.2.1 Expression of GPCR43 in 6 CRC cell lines

GPCR43 was present in 3 of 6 CRC cell lines tested, namely HT29, T84 and CaCo2 cells, but not in HCT116, SW480 or LIM1215 cells. It was intriguing to observe that although all cell lines tested in the current study possessed a copy of the GPCR43 gene, only 3 of the cell lines expressed the receptor. The absence of receptor expression was consistently observed for HCT116 cells where expression was also absent despite changes to cell confluence (Chapter 6) or nutrients (BuA and glucose) in the cell media (Chapter 7). Since the commencement of this study, a number of other studies have reported the expression of GPCR43 in the colonic tissue of mice (Dass et al., 2007), rats (Karaki et al., 2006) and humans (Karaki et al., 2007), thus confirming the results obtained in this study.

10.2.2 Expression of GPCR43 in patient samples

In the 3 cell lines positive for GPCR43 expression the level of receptor expression varied markedly. A similar result was also observed in the human patient samples (Chapter 9) where the cancerous biopsies showed a large variation in the expression of GPCR43. Interestingly, the expression of GPCR43 in the normal patient biopsies did not fluctuate greatly and was consistently lower than in the cancerous samples. The limitations of this pilot study have been clearly outlined in Chapter 9, and although no conclusions can be extrapolated from these results, the outcome of this pilot study clearly warrants further research.

Currently there are no explanations for why the expression of GPCR43 fluctuates so greatly between the HT29, T84 and CaCo2 cell lines or between patients with CRC. Nor is there an explanation for why HCT116, SW480 and LIM1215 cells do not express GPCR43 despite possessing a copy of the gene. It can, however, be determined from the experimentation conducted that the regulation of GPCR43 expression occurs at the mRNA level. It is also possible that post-transcription regulation also occurs and although investigation of the GPCR43 protein was of interest, experimentation was not successful in this area. The observation of GPCR43 regulation in CRC cell lines and in patient samples is, however, interesting and with further investigation may provide a greater insight the function of GPCR43. Approaches that may be taken for this work have been included in this Chapter.

10.2.3 Regulation of expression occurs with cell confluence and altered availability of nutrients

The expression of GPCR43 was tested at 85% confluency for all cell lines as previous studies had indicated cell culture confluence can effect the expression of some genes. In the current study the expression of GPCR43 was also shown to be modulated by cell confluence (Chapter 6) with a large, but insignificant, increase in GPCR43 at 80-90% cell confluence. This indicates the potential importance of investigating GPCR43 expression at a consistent confluence. The expression of GPCR43 was also shown to be modulated with changes to BuA and glucose concentrations in the extracellular environment.

The experimentation conducted and described above addresses the first aim described in this Chapter by demonstrating that GPCR43 is expressed in colonic tissue. Furthermore, the results also demonstrate that the GPCR43 expression may be modulated by several factors including cell confluence, altered nutrient availability and the development CRC.

10.3 The possible role of GPCR43 as an extracellular BuA sensor

GPCR43 expression was demonstrated in rats to change in response to nutritional status (Hong et al., 2005) and has since been hypothesised to be a nutrient sensor by a number of groups (Brown et al., 2005) (Covington et al., 2006) (Karaki et al., 2006). In the current study a similar hypothesis was presented and investigated by:

1. Determining whether GPCR43 expression was modulated by changes to BuA and glucose availability in the culture media.
2. Investigating the proliferative and apoptotic response of HT29 cells following addition of BuA in high and low glucose media.
3. Examining the expression of GPCR43 on HT29-R cells.

10.3.1 Expression of GPCR43 is mediated by nutrient availability

The first evidence to suggest that GPCR43 may have a role as a nutrient sensor was observed in Chapter 6 where a trend for increased expression of GPCR43 at 80-90% cell confluence compared with both 20-30% and 40-50% cell confluence was observed. There are a number of possible explanations, which have already been presented in that Chapter. At this confluency, cells had been proliferating for 72 hours and it is possible that the nutrients and energy sources in the cell media were depleted through both the time the cultures were in this media and as a response to the greater number of cells in the cultures. This could be tested by replenishing the media after this time, as nutrients would again become available and this may alter GPCR43 expression. As a response to the decreased availability of energy it is possible that the up-regulation of GPCR43 was a metabolic response of the cell attempting to detect or supply alternate energy sources.

The “GPCR43 as a BuA sensor” hypothesis was investigated in more detail in Chapter 7 where the expression of GPCR43 was measured following changes to glucose and BuA concentrations in the extracellular environment. GPCR43 expression was up-regulated in low glucose environments compared with high glucose environments in the absence of BuA, which supports this hypothesis. Up-regulation of GPCR43 in low glucose environments is likely to be a metabolic response of the cells arising from the reduced availability of the glucose energy source. It was hypothesised that due to the decreased glucose concentration, it is possible that there will be an up-regulation of sensors or transporters that have the potential to detect or supply energy from glucose or alternate energy sources. Other studies have demonstrated results which support this finding and the conclusion (Boren et al., 2003) (Cuff et al., 2002) (Li et al., 2006) and have been discussed in detail in Chapter 7. However, the influence of BuA exposure, chronic or acute, was of greater significance and influence than that of glucose.

GPCR43 expression is down-regulated in HT29 cells following BuA exposure irrespective of whether high or low glucose concentrations are present. Other studies

have shown that following prolonged exposure to ligand, GPCRs undergo desensitisation that can eventually lead to the down-regulation of the receptor at the mRNA level. The results from Chapter 7 suggest that exposure of the HT29 cells to 5mM BuA was sufficient to induce receptor desensitisation and induce down-regulation of GPCR43. This provides evidence to suggest that GPCR43 is acting as an extracellular BuA sensor in the colon, as in the presence of abundant ligand (in this case BuA) the receptor expression is down-regulated. This could be investigated further with time course experiments to determine when the receptor is down-regulated.

As described in Chapter 7, many CRC tumours can undergo a number of cellular processes to enable the development of resistance to chemotherapeutic agents (Cusack, 2003). This acquisition of resistance to apoptosis or chemotherapeutic drugs is considered to be a major obstacle in cancer treatment (Olmo et al., 2007). The expression of GPCR43 in cells resistant to BuA (HT29-R cells) was measured in high and low glucose concentrations. HT29-R cells showed low levels of GPCR43 expression, which were comparable to normal HT29 cells that had been treated with an acute dose of BuA (5mM, 48hours). This result provides further evidence that GPCR43 is down-regulated in response to ligand exposure occurs irrespective of whether BuA exposure is short or prolonged. It can be speculated that this observation is consistent with a role of GPCR43 in sensing the extracellular BuA environment.

10.3.2 The proliferative and apoptotic response of HT29 cells to BuA is altered by the availability of glucose

In Chapter 7, HT29 cells were exposed to a range of BuA concentrations for 24 and 48 hours in media containing either high or low glucose concentrations to determine the effect that altered nutrient availability would have on proliferation and apoptosis. After 24 and 48 hours there was significantly less proliferation in low glucose media alone compared with high glucose media alone. At 48 hours, BuA concentrations of 2.5mM-5mM enhanced cell proliferation in low glucose media compared with high glucose media. As described in Chapter 7, other studies have demonstrated a reversible adaptation of HT29 cells to different nutrient environments, and have proposed that a shift from BuA-driven to glucose-driven metabolism occurs with malignancy. Consequently, the results obtained in the current study could also demonstrate an adaptation of HT29 cells to altered nutrients in the media as previously discussed in greater detail in Chapter 7.

Similarly, the apoptotic response of HT29 cells exposed to BuA in media containing high and low glucose concentrations was examined. At the 24 hour time point, differences in BuA-induced apoptosis were observed at the 2.5mM concentration of BuA, with significantly less apoptosis observed in cells exposed to low glucose media. The magnitude of the apoptotic response, however, was substantially less than the apoptosis induced at the 48 hour time point so this statistically significant response was actually relatively small. After 48 hours, there were minimal differences in the levels of BuA-induced apoptosis were observed in both high and low glucose treatments at most BuA concentrations. Although there was a significantly higher level of BuA-induced apoptosis observed in low glucose media with 20mM BuA concentration, this concentration of BuA is high for *in vitro* studies and may not be physiologically relevant. For a full discussion of this result refer to Chapter 7.

10.3.3 Summary

The results summarised in this Chapter demonstrate that GPCR43 expression may be modulated by the availability of glucose and BuA in the extracellular environment. Furthermore, HT29-R cells showed comparable levels of GPCR43 expression to normal HT29 cells that had been treated with an acute dose of BuA. The BuA-induced proliferative response of HT29 cells in low glucose media showed a significantly different profile to HT29 cells in high glucose media, with low concentrations of BuA in low glucose media promoting proliferation. It was hypothesised from these results that the HT29 cells were able to undergo adaptation to utilise the BuA as an energy source. Since the proliferative response of cells to BuA was modulated by glucose availability, and the expression of GPCR43 was also regulated by changes in glucose concentration, these results may provide some evidence that GPCR43 acts as a BuA sensor and regulates BuA-induced changes to cell proliferation and apoptosis. To investigate this idea more conclusively, a number of functional studies were conducted to elucidate the role of GPCR43.

10.4 The possible function of GPCR43

It was hypothesised in the current study that BuA may exert its effects on apoptosis and proliferation via a cell surface receptor and that GPCR43 may modulate this process. A number of experiments were conducted to verfiy the function of GPCR43 in this thesis and include:

1. Investigating the possible role of G-proteins in BuA-induced apoptosis and proliferation using the G-protein inhibitors PTX and GP2A in Chapter 3.
2. The use of RNAi knockdown of GPCR43 expression to conclusively determine the role of this receptor in BuA-induced decreases in proliferation and increases in apoptosis.
3. The potential of GPCR43 to mediate cell differentiation as described in Chapter 6.
4. Measuring the proliferative and apoptotic response of HT29-R cells compared to normal, non-resistant HT-29 cells.

10.4.1 GPCR43 expression is not vital to BuA-induced changes to proliferation and apoptosis

The magnitude and pattern of response induced by BuA was unique to each of the 6 CRC cell lines tested in Chapter 3. Cells that did not express GPCR43 expression underwent similar BuA-induced changes to proliferation and apoptosis as those cells with expression of this receptor. Furthermore, the GPCR43 positive T84 cells were largely resistant to BuA-induced decreases in proliferation and increases in apoptosis. These suggest that neither the pattern or magnitude of BuA-induced changes to proliferation or apoptosis are exclusively linked to GPCR43 expression. However, BuA-induced apoptosis was shown to be mediated via GPCRs in HT29 cells but not HCT116 cells using the G-protein inhibitors PTX and GP2A (Chapter 3). These results show that G-proteins have the potential to mediate BuA-induced apoptosis, but are not required for apoptosis to occur.

To conclusively demonstrate if GPCR43 mediated in BuA-induced changes to apoptosis and proliferation, knockdown of the receptor expression was conducted using RNAi. The findings from this study indicate that high levels of expression of GPCR43 is not absolutely required for BuA-induced changes to apoptosis or proliferation. There are, however, several limitations to this experimentation. It is possible that there was an insufficient reduction in GPCR43 expression to achieve any alterations in the functional assays, although other studies have demonstrated physiological function of GPCR43 using similar levels of knockdown (Hong et al., 2005). Furthermore, exposure of cells to transfection reagent and siRNA molecules induced non-specific effects following exposure to mid-high concentrations of BuA. Possible alternative experimentation was presented in Chapter 8 which may overcome these non-specific effects. Finally, the addition of BuA to the functional proliferation and apoptosis assays induced a down-

regulation of GPCR43 which was greater than the knockdown achieved using RNAi. Since BuA must be added to investigate BuA-induced changes to the proliferative and apoptotic response of CRC cells, the expression of GPCR43 was down-regulated to similar levels of expression by BuA exposure in all treatment groups including the controls. This finding presents a significant issue as it demonstrates that the role of GPCR43 in BuA-induced changes cannot be determined using RNAi. Future experimentation investigating the role of GPCR43 in BuA-induced decreases in proliferation and apoptosis may be more successful with the use of an animal GPCR43 knockout model. However, as outlined in Chapter 7, the use of an animal knockout model may present other challenges limiting to conclusive experimentation.

The final conclusion from the proliferative and apoptotic experiments conducted in this thesis is that GPCR43 is not likely to be vital to BuA-induced changes to apoptosis and proliferation, for cells not having for GPCR43 expression can still undergo BuA-induced changes. On the other hand, a clear role of G-proteins in the apoptotic response were observed, which was evident by the inhibition of the BuA-induced apoptotic response of the GPCR43 positive HT29 cells but not the GPCR43 negative HCT116 cells with G-protein inhibitors. Knockdown studies were attempted to elucidate the function of GPCR43 further. These experiments have provided the opportunity to explore in more detail the influence of GPCR43 function, however the results were inconclusive.

10.4.2 The trend for increased GPCR43 expression induced by cell confluence is not linked to HT29 cell differentiation

Several studies have linked an increased differentiation status of cells with an increased cell confluence (Lai and Pittelkow, 2004) (Comalada et al., 2006) (Su et al., 2007) (Poumay and Pittelkow, 1995), and as described in Chapter 6, GPCR43 has also been implicated with cell differentiation (Senga et al., 2003) (Hong et al., 2005). In Chapter 6, the expression of GPCR43 tended to be up-regulated in cells grown at 80-90% cell confluence but this difference was statistically insignificant. It was, therefore, of interest to investigate if this increased GPCR43 expression was linked to the enhanced differentiation of HT29 cells as a result of greater cell confluence.

Results from Chapter 6 demonstrated no change in the differentiation status of HT29 cells with increasing cell confluency, and therefore suggested that the increase in GPCR43 expression observed at the 80-90% confluence is not related to HT29 differentiation.

Other groups have examined the link between GPCR43 and differentiation (Senga et al., 2003) (Hong et al., 2005), and show a relationship, however, in these studies differentiation was induced by an external agent. In the current study, investigation of the differentiation of HT29 cells using BuA was also conducted. The results from this work clearly show an increase in the differentiation status of HT29 cells following 5mM BuA exposure for 48 hours. To elucidate whether the enhanced differentiation induced by BuA was mediated by GPCR43, further studies would be required, such as RNAi knockdown. However, as described in the previous section, RNAi knockdown was utilised to measure the role of GPCR43 in BuA-induced decreases in proliferation and apoptosis with limited success. Therefore, the role of GPCR43 in BuA-induced differentiation should be investigated once a GPCR43 knockout model or other suitable experimental procedure is established.

10.4.3 GPCR43 and the acquisition of resistance to BuA

To investigate the possible role of GPCR43 in the acquisition of BuA resistance the expression of GPCR43 and the proliferative and apoptotic response to BuA exposure was investigated in both normal HT29 and HT29-R cells. In Chapter 7, the addition of BuA to the media of HT29 cells induced a down-regulation of GPCR43, however, the cells still underwent changes in proliferation and apoptosis. In HT29-R cells, which were grown in the constant presence of BuA, the expression of GPCR43 was demonstrated to be comparable to the down-regulated GPCR43 expression seen in normal HT29 cells exposed to BuA. However, in the HT29-R cells, BuA only induced minor changes in proliferation and apoptosis compared to normal HT29 cells. These results suggest that GPCR43 is possibly not involved in the acquisition of BuA resistance as HT29-R cells and HT29 cells treated with BuA have highly similar expression of GPCR43 yet profoundly different apoptotic and proliferative responses to BuA exposure.

10.5 Future Directions

To gain a more comprehensive understanding of the role of GPCR43 future studies would need to focus on:

1. Investigation into why the expression of GPCR43 is variable in CRC cell lines and in patient CRC samples. A large scale study on the expression of multiple genes, including GPCR43, in biopsies of normal and cancerous colonic tissue is currently being conducted by Dr. Caroline Kerr. This study will enable the

expression of GPCR43 to be correlated with the development and progression of CRC, and may provide further insight into the function of this receptor.

2. The role of GPCR43 as a BuA sensor utilising high and low glucose media environments. In the current study, GPCR43 was shown to not have a vital role in the mediation of BuA-induced apoptosis and proliferation. However, the interesting results obtained by investigating GPCR43 expression in high and low glucose environments may still suggest a role for GPCR43 as a BuA-sensor, which will exert other effects upon receptor activation. To investigate this further it would be ideal to culture cells in completely glucose-free media to determine the effects of BuA in this system.

3. The generation of a GPCR43 knockout mouse model to enable the function of GPCR43 to be investigated more conclusively. Although there are limitations to these models, it is likely that these experiments would provide further information to elucidate the function of GPCR43.

4. The creation of an antibody to the GPCR43 receptor to measure protein expression levels. As described previously this thesis it would of interest to measure the protein expression of GPCR43. Although mRNA expression can change and can provide a certain amount of information relating to the changes in receptor expression and function, it does not always correlate to protein expression (Chambers, 2002). Attempts to measure the GPCR43 protein were conducted in the current study using FACS and Western Blotting with 6 commercially available monoclonal antibodies (AbCam, Aust), and should be continued in the future if more specific antibodies can be sourced or generated.

5. Extensive evaluation of GPCR43 has been conducted as a part of this thesis due to its predicted involvement in BuA mediated effects on CRC cell lines. However, it would be of equal importance to investigate the role of other receptors in this family which bind BuA, in particular GPCR41.

10.6 Conclusion

The major aims of this study were summarised in Chapter 1 and are as follows:

1. Determine if GPCR43 is present on a range of CRC cell lines derived from adenocarcinoma, carcinoma and metastatic disease.

2. Investigate whether the expression of GPCR43 is altered with various changes to the cellular environment.
3. Determine if GPCR43 expression is modulated by disease state using biopsies from patients' normal and malignant tissues.
4. Investigate whether GPCR43 is involved in mediating BuA-induced decreases in proliferation and increases in apoptosis.

The results from this thesis have provided the following responses to those aims:

1. GPCR43 expression was detected on 3 of the 6 CRC cell lines tested in this study, although no correlation was observed with the progression of CRC using cells lines derived from adenocarcinoma, carcinoma and metastatic disease.
2. GPCR43 expression is significantly modulated by acute and chronic changes to extracellular BuA concentrations and to a lesser extent glucose concentrations. This was considered to be one of the most interesting findings in this study. A trend for the modulation of GPCR43 expression with changes to cell confluence was observed at 80-90% confluence compared to 20-30% and 40-50% confluence.
3. In the small pilot study conducted in this Thesis, a difference in the expression of GPCR43 was observed in human cancerous biopsies compared with normal biopsy samples. Although this presents an interesting finding, the results of this pilot study must be interpreted with caution due to limitations of the study which have been described in detail in Chapter 9.
4. The results of this study did not demonstrate a tight relationship between GPCR43 expression and BuA-induced changes in apoptosis and proliferation. However, an influence of G-proteins on the magnitude of BuA-induced apoptosis was seen in HT29 cells which expressed the highest levels of GPCR43.

Finally, the results have also provided some support for the role of GPCR43 as a sensor for BuA. The results also indicated the potential importance of BuA as a physiological regulator, and provide further understanding of its action in CRC cells. With further experimentation it is hoped that the function of GPCR43 will be confirmed and the pathways which mediate BuA-induced effects on proliferation and apoptosis are elucidated.