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Anti-Proliferative and Cytotoxic Effects of CBD and CBDA in Human Colorectal Cancer

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Table of Abbreviations

CaCo-2	Heterogeneous human epithelial colorectal adenocarcinoma cells
CBD	Cannabidiol
CBDA	Cannabidiolic Acid
$CB_{1/2}$	Cannabinoid Receptor
CRC	Colorectal Cancer
ECS	Endocannabinoids System
GI	Gastrointestinal
$GPR_{55/18}$	G Protein-coupled Receptor 55/18
$5\text{-}HT_{1A}$	5-Hydroxytryptamine Receptor 1A
IBD	Inflammatory Bowel Disease
$PPAR_{\alpha/\gamma/\delta}$	Peroxisome Activated Receptor α/γ/δ
$TRPV_1$	Transient Receptor Potential Vanilloid 1
THC	Tetrahydrocannabinol

Abstract

Introduction: Colorectal cancer (CRC) is one of the most prevalent cancers in Europe and fourth most common globally (Boyle and Langman, 2000). The phytocannabinoids, cannabidiol (CBD) and cannabidiolic acid (CBDA) are non-psychoactive cannabis derived lipid compounds which are being increasingly investigated for their anti-cancer effects. CBD and CBDA have proven beneficial in multiple *in vitro* human cancer cell lines exhibiting anti-inflammatory, anti-proliferative and cytotoxic effects. We aimed to explore the potential application of these compounds and their effects in a CRC cell and human explant tumour model.

Methods: Exploration of the anti-proliferative and cytotoxic properties of CBD and CBDA was conducted in a CaCo-2 human colorectal carcinoma cell line followed by a CRC explant model using human tissue. Metabolic activity was quantified using

the resazurin salt assay over 72 hours. Interleukin expression was analysed by enzyme-linked immunosorbent assay. A BCA assay was used for the analysis of total protein content and lactate dehydrogenase used as a method of assessing cell viability. Receptor antagonists were used to explore the mechanisms of the anti-proliferative and cytotoxic effects. Cytokine and protein responses were measured through enzyme linked immunosorbent assaying (ELISA).

Results: We report significant anti-proliferative and cytotoxic effects in CaCo-2 cells at a concentration of 70µM in proliferating cells and 50 µM in confluent cells. CBDA exhibited higher anti-proliferative potency than CBD exerting effects at 30µM but demonstrated only weak cytotoxic effects. CBDA and CBD increased extracellular LDH and decreased total protein content of CaCo-2 cells indicating cytotoxicity. Combination treatments of CBD and CBDA exhibited no increase in efficacy. Our explant model exhibited several promising anti-cancer effects, however these studies comprised of only an n=2 due to time constraints and delays. The GPR_{55}, 5-HT_{1A} PPARδ receptors were implicated in the mediation of CBD and CBDA's anti-proliferative and cytotoxic effects.

Conclusions: CBD and CBDA show great efficacy as anti-cancer compounds. We show that the anti-proliferative and cytotoxic effects appear to be mediated in a GPR_{55}, 5-HT_{1A} PPARδ sensitive manner. For the first time, we demonstrate that CBDA appears to be a more potent anti-proliferative whilst CBD exhibits higher cell cytotoxicity. Combination treatments of CBD and CBDA exhibit no augmentation in effect.

1 Introduction

Cannabis preparations have been medicinally utilised for millennia with ancient civilisation globally using the plant both medicinally and recreationally. In recent years, the demand for access to cannabis-based medical products (CBMP's) has drastically increased. As to has the need for clinical research and evidence to support the use of these medicines (Ben Amar, 2006; Pisanti *et al.*, 2017). Cannabis is widely and crudely used by patients suffering from various chronic and terminal illnesses (Webb and Webb, 2014a; Martell *et al.*, 2018). The uses of cannabis vary significantly. Used in many cases by those living with chronic pain but also utilised by sufferers of spinal cord injuries and movement disorders (British Medical Association., 1997; Kumar, Chambers and Pertwee, 2008). Cannabis use amongst cancer patients is of growing significance. Swathes of anecdotal evidence argues that cannabis possesses anti-cancer properties for some time now (Mouhamed *et al.*, 2018). These anecdotal evidence is well founded, with numerous cancer cell lines having provided promising data to support these previously unsubstantiated claims (Ramer *et al.*, 2012; Honarmand *et al.*, 2018). Amongst the many forms of modern cancers, colorectal cancer (CRC) is one such form that is particularly prevalent (Munkholm, 2003). CRC's currently lack non-invasive therapeutic option and as are likely to benefit from the development of novel cannabis based treatment strategies (Namani *et al.*, 2017; Lui, Tsoi and Sung, 2018). There is tremendous potential for cannabinoid medicine in colorectal cancers, this research aims to developing a deeper understanding of the physiological mechanisms underlying the anti-cancer effects of cannabis and its derivatives.

1.1 Physiology of the Gastrointestinal Tract

The CRC is a disease which affects the Gastro-Intestinal (GI) system, which is comprised of a series of organs spanning from mouth to anus. It is divided into two parts, the luminal GI, and the hepato-biliary-pancreatic GI. The luminal GI is muscular tube, comprised of the, pharynx, oesophagus, stomach, small and large intestine as well as the anus, totalling approximately five metres from start to finish (Leung, 2014). The hepato-biliary-pancreatic GI consists of the salivary glands, pancreas, gallbladder, and liver, which release digestive secretions into the lumen of the gut (Leung, 2014). These systems operate in a coordinated manor to digest, absorb and distribute nutrients throughout the body, utilising both chemical and mechanical absorption. The gastrointestinal tract is responsible for the digestion of food, absorption of nutrients and the elimination of waste product. Homeostasis of the gastrointestinal system is regulated through a series of intrinsic and extrinsic neuronal and endocrine mechanisms. These mechanisms are responsible for the coordination of gut motility, digestion and absorption and are integral to the regulation of hunger and feeding behaviours (Keshav and Bailey, 2013). The GI tract is separated into key locations by smooth muscle structures called “sphincters”, which manipulate luminal contents in a highly coordinated manner (Leung, 2014). One of the gastrointestinal tracts key functions is to form a selective barrier between the external environment within the contents of the lumen and sterile blood of the host (Karwad *et al.*, 2017). This selective absorption can occur through para-cellular passage, active or passive transcellular transport and endocytosis allowing selective permeability to the desired nutrients (Balimane *et al*., 2000).

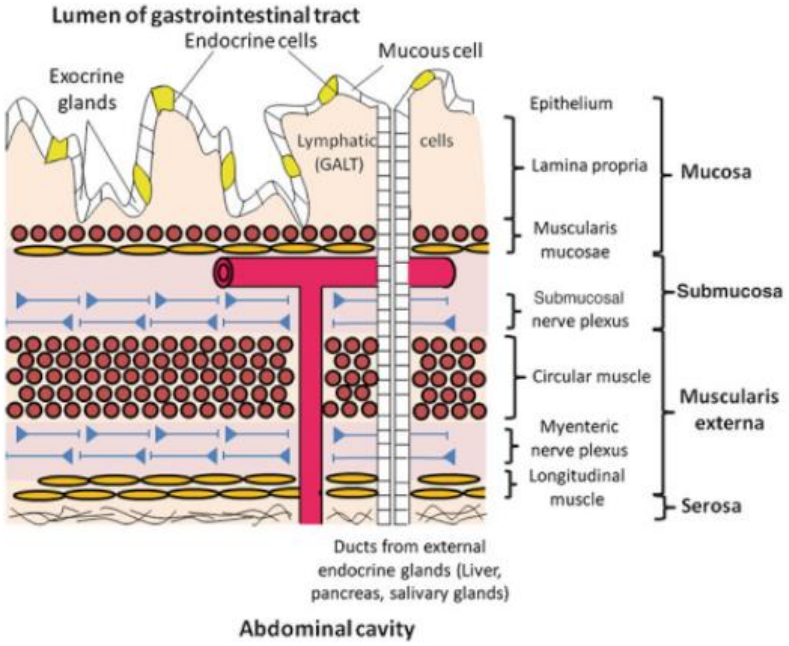


Figure 1. Physiological appearance of a longitudinal section of the digestive tract wall. GALT = gut associated lymphoid tissue (modified from Widmaier *et al.*, 2008; Leung, 2014)

The lumen of the GI tract is comprised of four discernible tissue layers; the serosa, muscularis, submucosa and mucosa, each of which have a specialised function in gastric function (***Figure 1***). The outermost layer, the serosa, is made up of a fibrous connective tissue consisting of elastin and collagen fibres, lined by a squamous epithelium, this structure supports the abdominal cavity, connecting to the abdominal wall (Leung, 2014). The peristaltic movements of the gut are generated by contractions of the muscularis layer, this comprises inner circular and longitudinal muscle fibres. The muscularis also accommodates the mesenteric nerve plexus, a prominent network of nerve cells, located between the circular and longitudinal smooth muscle layers (Leung, 2014). The second most inner layer is

the submucosa, this region contains, major lymphatic vessels and vasculature, along with a plexus of nerve cells, called the submucosal nerve plexus. The innermost layer, and area of interest in this study is the mucosa. The mucosa is made up of several cell types including an epithelium, *lamina propia* and *muscularis mucosae* (Leung, 2014). The structure of the lumen facilitates a mutualism of bacteria and host in gut, comprising what is known as the microbiome. Our knowledge of the gut microbiome has been steadily increasing, however there are currently 10x the number of microbial cells in the gut than in the whole human body leaving a great deal to be elucidated, this microbiome totals roughly 100 trillion microbes from as many as 5,000 different species, weighing approximately 2 kilograms (Ferranti *et al.*, 2014). The vast majority of the 10–100 trillion microbes in the human gastrointestinal tract live in the colon (Turnbaugh *et al*, 2007). The maintenance of the gut microbiome and selectivity of the gut are integral to the overall function of the bowel. Dysregulation of this delicate biology, due to a variety of factors, may lead to sustained inflammation within the gut which is thought to be the underlying cause of chronic inflammatory bowel conditions such as IBD (Henderson *et al.*, 2011; Hold *et al.*, 2014). The importance of the microbiome homeostasis in colorectal cancer has only been recently appreciated, literature suggests that compromise of this homeostasis may be one of the underlying mechanisms of colorectal cancer pathogenesis (Hsiao *et al.*, 2008; Mager *et al.*, 2016).

1.2 Physiology of Colorectal cancer (CRC)

Colorectal cancer may arise in one of three ways. Either sporadically, due to environmental factors, or as a result of genetic susceptibility to oncogenes or via the inheritance of familial genes which are present in several family members. The progression of colorectal cancer is typically sporadic in nature and accounts for approximately 70% to 75% of cases (Amersi *et al*., 2005). There are a multitude of other factors that can contribute to the pathogenesis of colorectal cancer, which are classed as either modifiable or non-modifiable risk factors (Amersi *et al*., 2005; Dyson and Rutter, 2012). Colorectal cancer patients exhibit a variety of non-specific symptoms including abdominal pain, fatigue, weight loss and rectal bleeding to note just a few (Hamilton *et al.*, 2005; Rasmussen *et al.*, 2015). One particular problem facing CRC management strategies is the inherent lack of none invasive methods, resulting in only a handful of therapeutic options being available to CRC patients. CRC Patients are currently have a limited selection of three chemotherapeutic agents, Antimetabolites (5-flourouracil), Alkylating agents and Topoisomerase Inhibitors (Warusavitarne and Schnitzler, 2007). Although chemotherapy is a viable and well tested treatment option, chemotherapeutics are not effective in all cases and often result in in numerous unwanted off target adverse effects (Ma *et al.*, 2011; Munker *et al.*, 2018).

A method for determining the staging for CRC tumours has been devised based on the three characteristic changes observed in cancer progressions. The tumour, node, metastasis (TNM) staging model is now the international standard for

colorectal cancer staging, and is endorsed globally by medical bodies, including the international union against cancer (UICC) (Sobin *et al.*, 2011).

1.3 Incidence and Mortality

Colorectal Cancer is a predominantly western form of cancer with growing global incidence rates with the highest prevalence seen in westernised regions such as Australia/New Zealand, Europe, and Northern America (Torre *et al.*, 2016). CRC is one of the most prevalent cancers in Europe and fourth most common globally (Boyle and Langman, 2000). Worldwide, CRC is the third most commonly diagnosed cancer in males and the second in females, with an estimated 1.4 million cases and 693,900 deaths occurring in 2012 (Ferlay *et al.*, 2015; Torre *et al.*, 2015). The incidence of CRC increased significantly in both males and females from 1983-87 to 1998-2002 (Center *et al*., 2009). Despite these alarming statistics, incidence rates can vary up to 10-fold globally depending on the degree of regional development and the extent to which the western culture has been adopted (Center *et al*., 2009; Ng *et al.*, 2017). As of 2014, CRC incidence rates within the United Kingdom were approximately 8.37 per 10,000 persons, a reduction from the figures recorded in the year 2000, a year where colorectal cancer affected 9.27 per 10,000 persons (Soriano *et al*., 2018). In recent years, Western countries, such as the UK, CRC incidence has stabalised, attributed primarily to the success of state sponsored screening programs, and a reduction in the prevalence of cancer associated risk factors (Torre *et al.*, 2015). The elevated risk of cancer to the UK population was highlighted in 2000 after the EUROCARE-4 report identified unexpectedly poor cancer outcomes for patients within the UK (Berrino *et al*.,2007). Since these findings, the UK has continued to rank highly in terms of total net expenditure on

health (TNEH) provision. However, when compared to countries of a similar TNEH, the UK still vastly under performs and ranks lower in all-cancer outcomes, highlighting weaknesses in the UK cancer management system (Baili *et al.*, 2015). The UK further under performs in terms of 5-year survival with a high rate of death close to diagnosis, 11.5% of colon and 5.4% of rectal died within a month of diagnosis (Downing *et al.*, 2013; Murchie *et al.*, 2014). This disparity highlights inefficiencies in the allocation of public health funding and management of patients within the UK cancer treatment system, with patients, in some cases, experiencing treatment delays of between 4 and 30 weeks and in some cases an excess of 34 weeks (Murchie *et al.*, 2014). Unsurprisingly, these diagnostic delays have been correlated to a higher mortality rate in patients with colorectal cancer, which is challenged by several other studies who argued that this is heavily dependent of several factors including staging tumour characteristics (Simunovic *et al.*, 2009; Tørring *et al.*, 2011; Murchie *et al.*, 2014). CRC persistence has been attributed the heightened prevalence of obesity, hypertension, atrial fibrillation and subsequent medications within the UK, all of which increase the likelihood of CRC development (Soriano *et al*., 2018). The prevalence of these comorbidities and medications account for poor survival outcomes of newly diagnosed patients, despite stabilising incidence rates (Soriano *et al*., 2018). Despite these screening successes, CRC incidence continues to increase globally in individuals under 55 years of age, and disproportionally affects those living in westernised nations. Greater focus is now being drawn to reducing the effect of risk amplifying lifestyle factors.

1.4 Risk factors

There are a variety of risk factors that increase the chances of incurring colorectal cancer, many of which are avoidable and genetically linked. The prevalence of these cancer increasing risk factors within the British population have been linked to the relatively poor cancer outcomes observed in the UK (Soriano *et al*., 2018). Some of these risk factors are unavoidable. Described as “unmodifiable” risk factors, these are inherent patient traits which cannot affect, but increase their chances disease risk. Unfortunately however, there are also a variety of non-modifiable risk factors which predispose a variety of large populations to a significantly heightened likelihood of disease development (Haggar and Boushey, 2009).

1.4.1Personal history of IBD

Inflammatory Bowel Disease (IBD) refers to a group of gastrointestinal disorders that cause chronic or recurring immune responses and inflammation of the gastrointestinal tract (Duerr *et al.*, 2006). Chronic inflammation of the bowel associated with IBD is strongly associated with CRC, first described as early as 1925 by Burrill Crohn (Crohn, 1962; Bernstein *et al.*, 2001) . This increased risk is dependent on the anatomic extent, severity and duration of bowel disease heavily influences the likelihood of developing CRC (Munkholm, 2003; Beaugerie and Itzkowitz, 2015). Despite Only 1-2% of all CRC cases being attributed to the presence of IBD, 15% of all deaths in IBD patients are attributed to CRC (Munkholm, 2003). Symptoms of IBD include pain and diarrhoea which stem from chronic irritation of the gut lining, this irritation, over time, progressively and irreparably impairs function, often leading to malabsorption and malnutrition in patients

(Langmead and Irving, 2008). IBD describes two prevalent chronic idiopathic conditions, Ulcerative Colitis (UC) and Crohn's Disease (CD). Crohn's disease and Ulcerative Colitis differ slightly, with UC being restricted to the colon, rectum and mucosa only, whilst CD can be transmural and affect any part of the gastrointestinal tract (Campieri, 2002). Ulcerative Colitis sufferers typically present with bloody diarrhoea, proctitis (Rectal bleeding and discharge), proctosigmoiditis (abdominal discomfort and mucus discharge, affecting the rectum and Sigmoid Colon), and pancolitis (effecting the entire colon) (Waugh *et al.*, 2013). Crohn's Disease, although presenting with the same abdominal pain, diarrhoea (mucus and blood absent) and weight loss, may be differentiated from UC by analysis of intestinal biopsies and the presence of epithelioid granulomas (organised collections of macrophages) (Freeman, 2007) . Although the exact aetiology of inflammatory bowel disease is unknown genetic studies have identified the IL23R gene among others as contributing to the development of IBD (Duerr *et al.*, 2006). IBD currently effects approximately 620,000 people within the UK and up to 2.5-3 million people across Europe, having a healthcare cost of €4.6-5.6bn per year (Burisch and Munkholm, 2013). Epidemiological suggests a global rise in the incidence of IBD, with the highest prevalence identified within the Caucasian populations of Western Europe and North America, but now rising sharply in developing countries (Goh and Xiao, 2009; Molodecky *et al.*, 2012). Studies have identified a global rise amongst continental ethnic groups with prevalence similarly growing across developing countries throughout Asia, South America and Africa (M'koma, 2013). Despite deficiencies in IBD data from some developing countries, IBD prevalence seems to correlate with industrialisation and westernisation, suggesting modern lifestyle

factors augment the distribution of IBD globally (Ng *et al.*, 2014). The urbanisation of societies has meant that factors such as dietary changes, antibiotic use and hygiene promotion have been identified as contributing factors to the global rise in Inflammatory Bowel Diseases (Ponder and Long, 2013; Ng *et al.*, 2014). In a Danish study consisting of 57,851 Irritable Bowel syndrome (IBS), a less severe and persistent for of IBD, researchers found a that within the first 3 months of IBS diagnosis, there was an 8 fold increase in the standardised incidence ratios (SIR's) of colon cancer and 5-fold increase in rectal cancer (Nørgaard *et al.*, 2011). Patients were observed over the subsequent ten years, during which time their SIR of CRC development was reduced to 0.95. This initial increase in risk was likely attributed to the diagnostic confusion perpetuated by the overlapping symptomology of the two pathologies. A similar paper in 2009 found that IBS patients were at significantly increased risk of detecting IBD among patients initially diagnosed as having IBS (García Rodríguez *et al*., 2009). Given the susceptibility of so such a large population to CRC, CRC will no doubt be a perpetual issue for global healthcare professionals.

1.4.2 Colonic Polyps

Polyps are benign or malignant noncancerous growths that can develop in the bowel lining over varying periods of time. Colonic polyps (adenomas) are commonly known to develop into adenomatous polyps which are known to be one of the major initiation sites of most colorectal cancers (Amersi *et al*., 2005). Adenomas are exceptionally common in the mature population with as many as 50% of over 50 year olds harbouring one or more adenomatous polyps (Schatzkin *et al.*, 1994). A

study investigating the risk associated with the presence of adenomas found that over a 20 year period there was a 25% chance of developing CRC at the site of adenoma (Winawer *et al.*, 2000). Several physiological characteristics also determine the future severity of CRC pathogenesis. Should polyps present with Villous features, high grade dysplasia, be of a particularly large adenoma size, or present in a group of ≥ 3 adenomas, there is significantly further risk of future colonic neoplasia (Calderwood *et al*., 2016). Worryingly, over recent years the presence of colon adenoma in the population has increased. In a 2016 study of the incidence of adenomatous polyps and CRC incidence, the rate of CRC over a 20 year period declined despite a significant increase in the prevalence of adenomas, 16.5% in 1990 to 32.7% in 2010 (Milovanovic-Alempijevic *et al.*, 2018). This finding highlights the maintained necessity of national screening programs such as the national faecal occult blood test screening programme which was implemented in England in June 2006 and in Scotland in June 2007 with the aim of reducing the number of incident cases through detecting pre-cancerous CRC adenomas. Screening measures such as these have demonstrated great effect in preventative medicine. In a study investigating the benefit of bowel screening measures in a population of 32, 702 US veterans, screening procedures were deemed to reduce the likelihood of CRC development by as much as 50% protecting participants for up to 6 years post screening (Müller and Sonnenberg, 1995).

Inherited Genetic Risk

CRC risk can be further heightened by the presence of multiple genetic factors that predispose at risk groups to cancer development. The prevalence of this genetic risk was highlighted in a Scandinavian study of 44,788 pairs of twins, heritable factors were deemed to contribute up to contribute as much as 35% towards the development of colorectal cancer (Lichtenstein *et al.*, 2000). A person is typically deemed at greater risk of CRC when a first degree relative has been diagnosed with colon polyps or cancer before the age of 60 (Amersi *et al*., 2005). This genetic risk is further augmented if relatives of patients were diagnosed at a young age young, those with two or more affected relatives (Johns and Houlston, 2001). CRC risk can be further heightened by the presence of multiple genetic factors that predispose at risk groups to cancer development. The prevalence of this genetic risk was highlighted in a Scandinavian study of 44,788 pairs of twins, heritable factors were deemed to contribute up to contribute as much as 35% towards the development of colorectal cancer (Lichtenstein *et al.*, 2000).A person is typically deemed at greater risk of CRC when a first degree relative has been diagnosed with colon polyps or cancer before the age of 60 (Amersi *et al*., 2005). This genetic risk is further augmented if relatives of patients were diagnosed at a young age young, those with two or more affected relatives (Johns and Houlston, 2001)

1.4.3 Diet, Smoking and Alcohol

Despite the significant genetic aspect of colorectal cancer, the overall risk of cancer can be reduced if certain lifestyle choices and dietary factors are avoided (Gunter and Leitzmann, 2006). Diet is a highly influential factor influencing the incidence of and likelihood of developing CRC. Factors such as high consumption of red meat and processed meats and high dietary fat intake have been linked to CRC when compared to a typical omnivorous diet (Amersi *et al*., 2005; Namani *et al.*, 2017). A growing body of evidence has demonstrated that obesity and a poor diet increase the overall risk of cancer by approximately 19% (Baena and Salinas, 2015). This again is rather worrying given that obesity is a growing health concern in the United Kingdom. The prevalence of obesity increased from 15% in 1993 to 27% in 2015 with 58% of women and 68% of men were overweight or obese (Niblett, 2015). Unfortunately, these figures are only set to rise with the next future generations expected to exceed current obesity rates. As of 2016 nearly 1 in 3 children in grade 6 were obese or overweight (Niblett, 2015). The increased risk associated with obesity is modifiable, regular physical activity can reduce risk by 24%, the consumption of fish, by 12%, and intaking 20 g/day of fibre associated with a 25% reduction of CRC risk (Baena and Salinas, 2015). Smoking and alcohol have been long associated with increased cancer risk. There is however some doubt surrounding their true contribution to colorectal cancer despite a large quantity of evidence supporting its association (Amersi *et al*., 2005). Though doubt remains as to their contribution, approximately 12% of colorectal cancer deaths are attributed to smoking. Both alcohol consumption and smoking are prevalent lifestyle factors

across global cultures despite growing education around the health effects of these actions. In men, a longer duration and a higher average consumption of alcohol is associated with an elevated risk of colorectal cancer (Cho *et al.*, 2015). In a Chinese study, participants with a weekly consumption of seven or more alcoholic beverages a week are 72% more likely to develop colorectal cancer than non-drinkers (Tsong *et al.*, 2007). Alcohol consumption (25–30 g/day) increases CRC risk and rather predictably the risk of CRC is further increased in smokers (Baena and Salinas, 2015). Though not the cause of all CRC, these lifestyle factors significantly contribute to CRC and should be avoided in those patients who are already predisposed to heightened risk.

1.5 Chronic inflammation and Colorectal Cancer Pathogenesis

Though in 95% of cases colorectal cancer are deemed to emerge sporadically, chronic inflammation of the gut has long been associated with colorectal cancer (Karin and Greten, 2005; Kraus and Arber, 2009; Triantafillidis, Nasioulas and Kosmidis, 2009; Watson and Collins, 2011). Inflammation is a complex biological process occurring in response to the detection of harmful stimuli within the body. Inflammation was initially described by Aulus Cornelius Celsus having four distinct cardinal signs, redness and swelling, heat and pain, with loss of function later being added (Tracy, 2006). In healthy humans, inflammation serves as a protective response that eliminates the initial cause of cell damage, removes damaged cells

and establishes the foundations for tissue repair (Granger and Senchenkova, 2010; Zweifach *et al.,* 2014). Although inflammation is a regenerative process, it may also be potentially harmful, damaging surrounding tissues (Lawrence and Gilroy, 2006). Inflammation can occur either acutely, or be exhibited chronically, with varying physiological effects. Chronic inflammation is relatively slow process compared to acute inflammation and develops over an extended period. This extended inflammatory period can lead to DNA damage in the long term, which when combined with the correct environmental and heritable factors can lead to the development of colorectal cancer. This extended DNA damage is one part of a multistep process which due to a variety of contributing factors eventually leads to the accumulation of genetic and epigenetic mutations (Herceg and Hainaut, 2007).

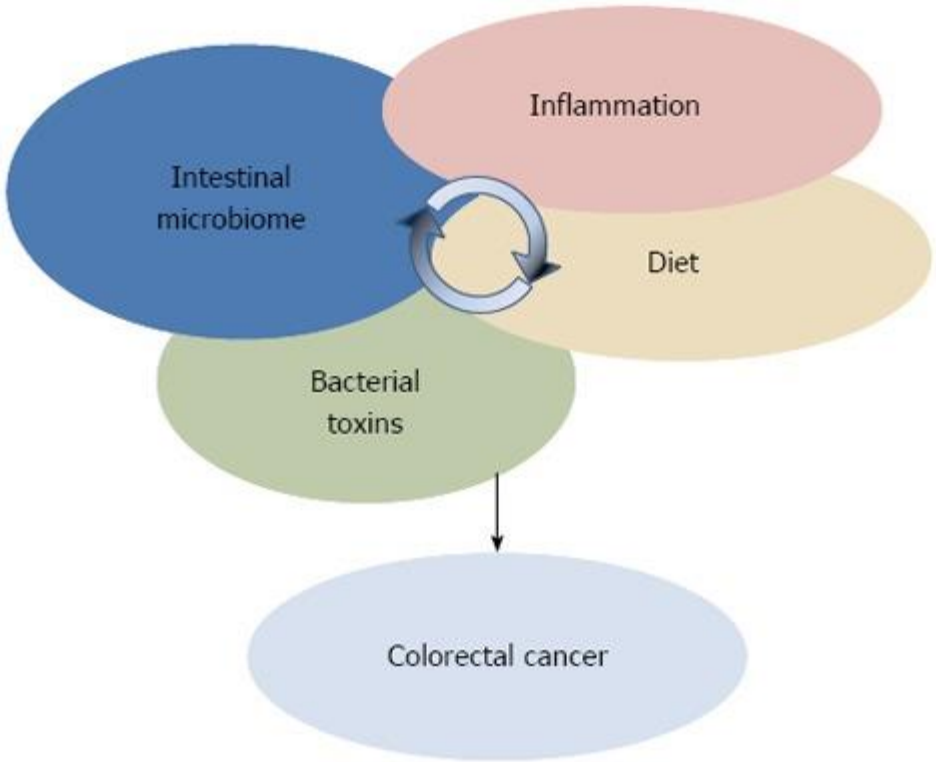


***Figure 2*.** A flow chart highlighting the overlap of multiple processes and factors in the formation of colorectal cancer (taken from Candela *et al.*, 2014).

Cells which become chronically inflamed attract monocytes, macrophages and lymphocytes to the site of tissue damage, prolonged exposure often leads to tissue injury and fibrosis, augmenting loss of function (Kono and Rock, 2008). Within the gut, the intestinal epithelial cells are the primary defence layer between the luminal contents of the colon and the internal body tissue (Coskun, 2014). In healthy individuals this barrier of cells regulates homeostasis between gut flora and the mucosal immune system (Roda *et al.*, 2010).These cells comprise an integral highly selective permeable membrane called the Intestinal epithelial barrier, which is composed of a thick mucus containing layer, a monolayer of intestinal epithelial cells and an underlying set of immune cells including, dendritic cells, mesenchymal cells, macrophages and lymphocytes (McGuckin *et al.*, 2009). Damage and dysfunction of the IEB is commonly identified in patients suffering from inflammatory bowel disease. Although the exact aetiology of IBD remains unknown, it is evident that genetic interactions with the immune system contribute to the autoimmune response associated with IBD (Ma, 1997). The dysfunction of the intestinal epithelial barrier in IBD has been attributed to sustained increase in pro-inflammatory cytokines, leading to impairment of tight junctions which inhibits barrier function (Roda *et al.*, 2010). Two major cytokines, IFN-γ and TNF-α act on the epithelium, disrupting the barrier and increasing permeability, this dysfunction can allow leakage of luminal contents triggering an immune response *(Clayburgh et al*., 2004). In patients with IBD, it is thought the body's immune system mistakes harmless gut flora for harmful invading bacteria and initiates an immune response, triggering inflammation (Duchmann *et al.*, 1995). Therefore, systems modulating the inflammation and permeability of the gut are of significant interest to

researchers exploring novel therapeutic products. To date, no cure exists for inflammatory bowel disease, with treatment focusing primarily on the reduction of the pathological inflammation, through a combination of corticosteroids, anti-inflammatories and antibiotics (Fakhoury *et al.*, 2014). Crucially, the maintenance of this chronically inflamed state, encourages mutations of mucosal DNA eventually culminating in damaged mucosal cells which acquire immunity to the human immune system (Seliger, 2005; Vinay *et al.*, 2015). Overexpression of pro-inflammatory bodies and the avoidance of apoptotic signals, ultimately promote unregulated proliferation (Candela *et al.*, 2014; Wang and DuBois, 2015). This inflammation causes several molecular changes triggering colorectal cancer by instigating dysplasia of the colonic mucosa which matures into colorectal cancer (Namani *et al.*, 2017). In sporadic colorectal cancers, this same process occurs, but instead, early adenomas undergo further tumorigenesis before forming cancerous polyps (Fornaro *et al.*, 2016). Increasingly, research exploring the impact of the gut microbiota and cancer progression has highlighted another contributing factor towards DNA damage. In a study exploring the structural variations in the microbiota gathered from colon of cancer patients and healthy individuals, found that that the biodiversity of cancerous individuals was reduced when compared to matched healthy tissue (Chen *et al.*, 2012). Literature suggests that the recruitment of tumour infiltrating immune cells, by pro-inflammatory bacteria incubates a neoplastic microenvironment contributing to cancer progression (Kostic *et al.*, 2013). Another aspect of bacterial contribution to colorectal cancer are a subset of healthy bacterial strains that contain toxin producing cells (Eckburg *et al.*, 2005). Bacterial strains such as *B. fragilis* toxin, have the capacity to promote colon

tumorigenesis by promoting expression of signal transducer and activator of transcription (STAT)-3, a T cell recruiter, this recruitment suggests a pro-carcinogenic role through a de-regulated inflammatory response (Wu *et al.*, 2009). Similarly, most toxic residues found in colorectal tumour tissue are derived from *Escherichia coli (E. Coli)* (Candela *et al.*, 2014). Under inflammatory conditions, there is an increased presence of these toxigenic *E. Coli* strains augmenting their potential to damage DNA or disrupt cell signalling (Schwabe and Wang, 2012). This contribution of multiple factors towards the progression of CRC including the compromise of the flora-host mutualism, make CRC a particularly challenging pathology (Candela *et al.*, 2014). The diversity of these cancer promoting factors in the progression CRC make this form of cancer difficult to manage on a clinical level but also in the human population. The breadth of contributing biological factors, accompanied by additional genetic and lifestyle factors validates the prevalence of CRC and why CRC remains a critical concern in population health. This initial inflammation is believed to trigger CRC tumorigenesis, by initiating the initial dysplasia of the mucosa, which accompanied by several molecular changes, results in colorectal cancer formation (Namani *et al.*, 2017)..

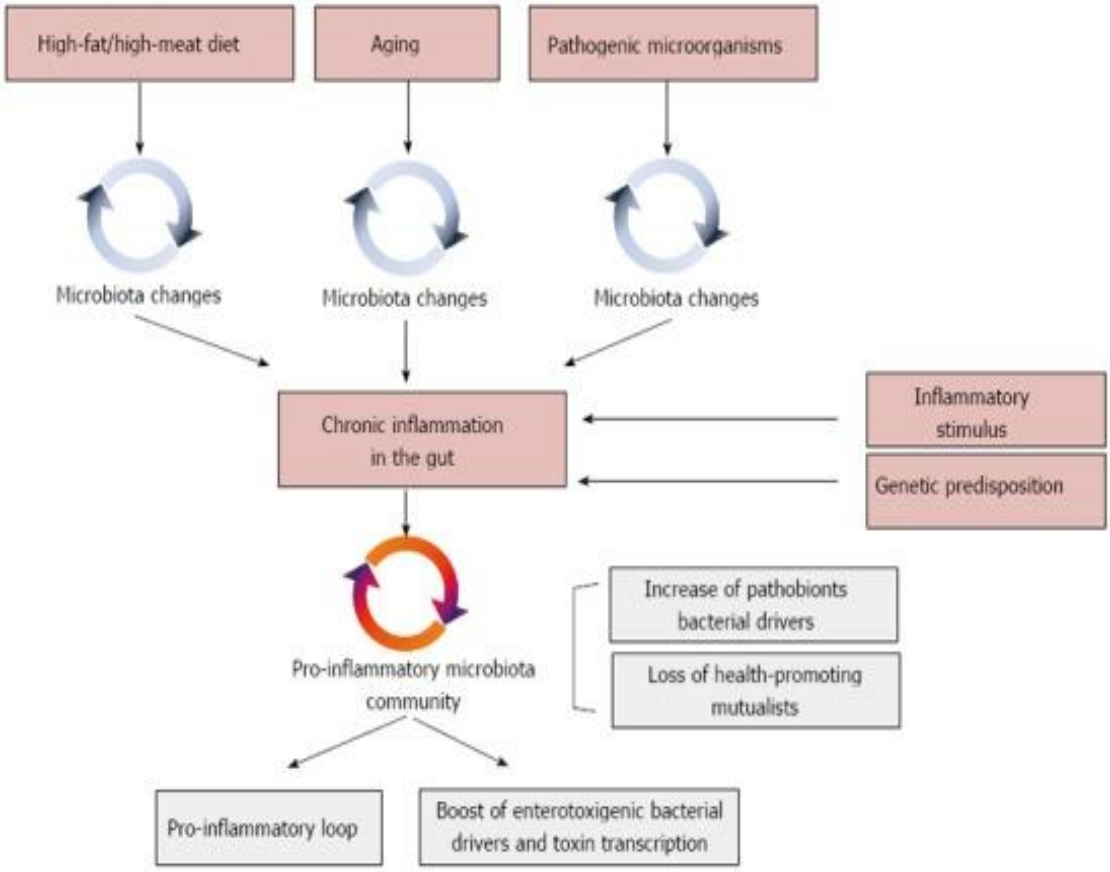


Figure 3. A schematic summarising the factors contributing to dysregulation of the gut microbiome that promote inflammation, leading to neoplasia, dysplasia, and tumorigenesis of colorectal cancer (taken from Candela *et al.*, 2014).

1.5.1 Molecular Alterations Associated with CRC

When investigating colorectal cancers, there are a variety of genetic components that must be considered when identifying potential efficacy of new anti-cancer drugs. Cancerous cells are well known to have altered gene expression, over expressing a variety of pro-inflammatory genes whilst under expressing cancer suppressing genes (Eberhart *et al.*, 1994; Kudryavtseva *et al.*, 2016). A compromised transcriptive process can overproduce a variety of pro-inflammatory

genes, which have been attributed to overexpression of several transcription factors (Vallabhapurapu and Karin, 2009). These transcriptive factors regulate the expression and production of pro-inflammatory cytokines, in turn, modulating the proliferation and survival of transformed cells (De Simone *et al.*, 2015). A key transcription factors regularly found over expressed in colorectal cancers is nuclear factor-kappa B (NF-kB) (Eiró and Vizoso, 2012). Recent data supports the role the NF-κB activated pathway in the initiation and propagation of colorectal cancers (Vaiopoulos *et al.*, 2013). Data suggests that up to 44.5% of the genetic components that make up the signalling pathways of NF-κB, are dysregulated in CRC's, relative to healthy mucosa (Lin *et al.*, 2016; Slattery *et al.*, 2018). Activation of this NF-κB pathway contributes to inflammation and tumorigenesis during tumour promotion and progression, by orchestrating the inflammatory process whilst participating in various steps of the cell cycle *(Zins et al., 2007; Vaiopoulos et al*., 2013). The tumorigenic involvement of NF-κB in colorectal cancer also extends to the metastasis of and invasion of cancer cells (Lin *et al.*, 2016). Given the widespread implication of the NF-κB gene in colon cancer, it a promising target for novel therapeutics and warrants further investigation (Hassanzadeh, 2011). Maintenance of NF-kB activity has been associated with signal transducer and activator of transcription 3 or the STAT3 transcription factor, another commonly over expressed signalling protein in colorectal cancer is the (Lee *et al.*, 2009). The STAT3 pathway is a well-known regulator of apoptosis, inflammation, transformation, proliferation, invasion, angiogenesis and metastasis of cancer (Bromberg and Darnell, 2000; Yu *et al*., 2009). In an *ex vivo* human study constitutive activation of STAT3 correlated with nodal metastasis and tumour stage

playing an important role in the tumorigenesis of colorectal carcinoma (Ma *et al.*, 2004). Further studies in 2007 demonstrated that both inactive (unphosphorylated) (UP-STAT3) and active (phosphorylated) (P-STAT3) STAT3 proteins were significantly increased in CRC (Lassmann *et al.*, 2007). This increased expression has also been linked to adverse inflammatory responses in hosts and an overall reduction in survival (Park *et al.*, 2017). Furthermore, inhibition of STAT3 with molecular inhibitors increased the sensitivity of the tumours to chemo-radiotherapy and has been found to down modulate proliferation and to induce apoptosis (Corvinus *et al.*, 2005; Spitzner *et al.*, 2017). This widespread involvement of STAT3 in the biological functioning of cancer cells makes it an interesting target of anti-cancer therapeutics. When investigating colorectal cancers, there are a variety of genetic components that must be considered when identifying potential efficacy of new anti-cancer drugs. Cancerous cells are well known to have altered gene expression, over expressing a variety of pro-inflammatory genes whilst under expressing cancer suppressing genes (Eberhart *et al.*, 1994; Kudryavtseva *et al.*, 2016). A compromised transcriptive process can overproduce a variety of pro-inflammatory genes, which have been attributed to overexpression of several transcription factors (Vallabhapurapu and Karin, 2009). These transcriptive factors regulate the expression and production of pro-inflammatory cytokines, in turn, modulating the proliferation and survival of transformed CRC cells (De Simone *et al.*, 2015).

The cyclooxygenase enzyme (COX) has also been heavily implicated in the development and progression of CRC (Sano *et al.*, 1995; Ferrandez, Prescott and Burt, 2003; Wang and DuBois, 2010). Two forms of COX enzyme exist, COX-1, expressed constitutively as part of normal cell health, and COX-2, an inducible

enzyme, mediates the inflammatory effects of COX and is induced and expressed in neoplastic growths (Ferrandez, Prescott and Burt, 2003; Koehne and Dubois, 2004). COX-2 expression is markedly higher in colorectal cancer tissue, this has been observed in cancer cells, inflammatory cells, vascular endothelium and fibroblasts when compared to normal colon tissue (Eberhart *et al.*, 1994; Sano *et al.*, 1995). In 1994, Eberhart *et al*, first documented that COX-2 expression was elevated in 85% of colorectal carcinomas and in approximately in 50% of colorectal adenomas, whilst the levels of COX-1 were unchanged. This over expression of COX-2 is induced by a breadth of growth factors, and pro-inflammatory cytokines *(Smith et al*., 1996; Smith *et al*., 2000). Cells containing constitutively expressed COX-2 have markedly increased invasive potential suggesting COX-2 may also have a role in the potentiation of metastasis *(Tsujii et al*., 1997). Molecules that reduce the activity and expression of COX-2 may be beneficial as adjuvant treatment in CRC management.

1.5.2 Interleukins and Cancer

It is becoming widely accepted that pro-inflammatory cytokines play an integral role in colorectal cancer development, with up to 20% of all cancers thought to arise in association with chronic inflammation (Grivennikov and Karin, 2011; Waldner *et al*., 2012). Cytokines are a family of signalling molecules that support autocrine and paracrine signalling between immune cells, these include, interleukins, Tumour Necrosis factors, interferons, colony stimulating factors and chemokines. The tumour-promoting role of interleukins (IL) has been of

tremendous interest in CRC research due to their role in the mediation of and maintenance of inflammation, a factor strongly linked to tumorigenesis (Kraus and Arber, 2009; Klampfer, 2011). Interleukins are typically synthesized by activated CD4 T lymphocytes, monocytes, macrophages and endothelial cells and are a common hallmark in colorectal cancer (Dembic and Dembic, 2015). Interleukin genes are known to play a significant role in the risk and survival of CRC, their tumorigenic role and pro-inflammatory properties highlighting their importance as a target molecule in cancer therapeutics (Grivennikov and Karin, 2011; Bondurant *et al.*, 2013). This study prioritises several interleukins which are of particular interest due to their implication in CRC. Interleukin (IL)-6 for example, is a pleiotropic cytokine, implicated in multiple biological interactions within the immune systems, haematopoiesis, inflammation and oncogenesis (Kishimoto, 2010; Barnes, Anderson and Moots, 2011). IL-6 is involved in the mediation of the acute inflammatory response, and is overexpressed in a variety of cancers (Gabay, 2006; Knüpfer and Preiß, 2007; Grivennikov and Karin, 2011). IL-6 can have a particularly detrimental impact in the gut as it promotes the accumulation of mononuclear cells (lymphocytes and monocytes) at the site of injury, this is promoted through continuous secretion of monocyte chemoattractant protein-1 (MCP-1), which can be blocked by anti-IL-6 receptor antibodies (Mudter and Neurath, 2007; Barnes *et al., 2011)*. Increased presence of IL-6 in blood serum has been linked to advanced disease staging and decreased survival in CRC (Waldner *et al*., 2012). In addition to its existence in serum, IL-6 expression is raised in CRC tumours compared to healthy tissue, which is associated with increased cancer cell invasion depth and lymph node metastasis (Zeng *et al.*, 2017). Activation of the STAT3 and NF-kB transcription

factors by IL-6 this group of proteins are a particularly promising target for novel anti-cancer drugs (Waldner *et al*., 2012).

Several other members of the interleukin family are of interest in CRC and are targets of this study. Interleukin-10 (IL-10) is an 18kDa anti-inflammatory cytokine produced by M2 macrophages, with both pro-tumour and anti-tumour properties, (Mosser, 2003; Dembic and Dembic, 2015; Abtahi *et al.*, 2017). Expression of this interleukin 10 in the sera of CRC patients was significantly lower than in control groups, a finding which has been correlated to dysplasia (Mumm and Oft, 2013; Abtahi *et al.*, 2017). IL-10 has a unique role in mediating COX-2 expression at a transcriptional level through the inhibition of NF-kB at two levels, by inhibition of its binding to DNA and suppression of IκB kinase activity which stimulates NF-kB (Appleby *et al.*, 1994; Schottelius *et al.*, 1999). Il-10's anti-tumorigenic role is solidified by discovery that deficient levels of IL-10 in the sera could be contributing to CRC carcinogenesis by inducing COX-2 overexpression (Abtahi *et al.*, 2017). Similarly to IL-6, the implication of IL-10 in other stages of inflammation and its role in tumour suppression make it a valuable marker in the study of CRC. Research led by Stanilov et al., detected significantly increased levels of Il-10 and IL-23 in the sera of CRC patients. Il-10 sera levels were highest in patients with stage IV CRC (Stanilov *et al.*, 2010).

Interleukin-17 (IL-17) is another cytokine of interest in this study. Produced upon activation by a group of pro-inflammatory helper T cells called TH17, IL-17 is a 32 kDa pro-inflammatory cytokine that stimulates COX-2 and production of IL-6 (Dembic, 2015). In 2011, Liu et al., demonstrated the increased density of IL-17

producing cells in colorectal tumour tissue compared to healthy background tissue. The heightened expression of IL-17 in CRC is associated with a significant reduction in survival rate, this overexpression may facilitate angiogenesis through activation of vascular endothelial growth factors (VEGF) (Liu *et al.*, 2011). In mouse models of CRC, ablation of IL-17 or its receptor confirmed its previously suspected tumour promoting role, communicating directly within transformed colonic epithelial cells facilitating early tumorigenesis(Wang, 2008). NF-kB activity is also triggered by the engagement of IL-17 and is linked to proliferation of tumorigenic enterocytes, with inhibition of IL-17 enhancing therapeutic responsiveness to chemotherapy. A study in 2013 demonstrated in human tissue, that IL-17A induced colitis-like mucosal damage could be attenuated by anandamide and cannabidiol (Harvey *et al.*, 2014). This same study found widespread IL-17A and IL-17C dispersion in human colonic mucosal epithelium and lamina propria. The intraepithelial presence of TH-17 cells (IL-17 producing) in CRC tissue correlated to an improved outcome in CRC patients (Amicarella *et al.*, 2017). This work by Amicarella *et al*, suggests there may be a dual role for IL-17 and the cells that produce it. Despite previous reports demonstrating the pro-inflammatory action of IL-17, the additional recruitment of beneficial immune cells, such as neutrophils and cytotoxic CD8+ T cells by IL-17, suggests this heightened expression of IL-17 may serve as a beneficial protective immune response to CRC cells (Amicarella *et al.*, 2017). In light of the widespread implication of interleukins 6, 10, and 17 in colorectal cancer, their potential as a therapeutic targets is of particular interest in relation to the role of cannabidiol in CRC (Wang, 2008).

1.5.3 VEGF and Cancer

It is widely understood that the growth of new blood vessels is integral to the survival and metastasis of tumours (Harmey, 2004). In healthy tissue, VEGF is produced in almost all cells in response to hypoxia, and stimulated in a variety of physiological functions including bone formation, haematopoiesis and wound healing (Ferrara *et al.*, 1996; Gerber *et al.*, 1999; Chintalgattu, Nair and Katwa, 2003; Harmey, 2004). VEGF, and its angiogenic prowess, are of growing importance in the study of colorectal cancer with heightened expression of VEGF in tumours being associated with an increased rate of angiogenesis, proliferation and metastasis (Takahashi *et al.*, 1995; Ahluwalia *et al.*, 2014; Sabnis *et al.*, 2015). The identification of VEGF as a promoter of metastasis and tumour angiogenesis, has led to its use as a target in the combating of CRC, with VEGF detection now widely being utilised as a clinical prognostic tool in CRC staging (Ellis *et al.*, 2000; Bendardaf *et al.*, 2008). VEGF expression has been shown to increase in response to chemotherapy and radiotherapy, which is thought to be a contributing factor in the protection of tumour cells from apoptosis (Harmey, 2004). Of note, is the stimulatory role of Interleukin-17 in VEGF production by cancer cells (Liu *et al.*, 2011). The importance of VEGF in CRC is highlighted by a statistic that approximately 50% of CRC tumours exhibit VEGF expression which is otherwise absent in healthy tissue (Choi *et al.*, 2008). This increased expressions is linked to advanced lymph node status and distant metastasis (Clasper *et al.*, 2008). Given this vast contribution of VEGF to tumour survival and progression in CRC, anti-VEGF

treatments offer another potential therapeutic target for anti-cancer drugs (Hu *et al.*, 2015).

1.6 The Endocannabinoid System (ECS)

Cannabis based medicines have been utilized for millennia to treat a multitude of physical disorders ranging from epilepsy to gastrointestinal disorders (Maurya and Velmurugan, 2018; Uranga, Vera and Abalo, 2018). In recent years the use of medicinal cannabis has become a subject of international debate, with global political powers now looking to re-evaluate their stance on the plant and its derivatives (Pertwee, 2014). Within the UK, calls for medicalisation of cannabis has provoked the British government to legislate for access to medical cannabis for UK patients from the 1st of November 2018.

Cannabis research has progressed at an exponential rate since the initial discovery of the first "cannabinoid" Δ^9-tetrahydrocannabinol (THC), with over a hundred other cannabinoids having been identified (Pertwee, 2014). Three forms of cannabinoids exist; synthetic cannabinoids produced pharmaceutically, phytocannabinoids derived from cannabis, and endocannabinoids, synthesized within the human body by local enzymes (Pertwee, 2014). Prior to the synthesis and identification of THC in 1964, little was known about the pharmacological actions of cannabis and cannabinoids (Gaoni and Mechoulam, 1964). Investigation into the mechanisms of THC function led to the discovery of cannabinoid receptor 1 (CB_1) (Devane *et al.*, 1988). The discovery of this novel receptor prompted the investigation into the internal ligands mediating the function of CB_1 in the human body, these studies identified the endogenous molecules now known as

endocannabinoids (Howlett *et al.*, 1990; Pacher *et al*., 2006). The cloning of the cannabinoid receptor 1 in 1990 by Matsuda *et al*. aided in the development of agonists to activate the receptor and antagonists to inhibit its function, facilitating research into the wider interactions of CB_1. These early breakthroughs led to the identification of the Endocannabinoid System (ECS), a series of endogenous lipid signalling molecules, their synthesising/degrading enzymes and, their corresponding receptors (Nagarkatti *et al*., 2009). Since it's discovery, the ECS has been implicated in a multitude of significant regulatory physiological processes including appetite, metabolism, pain sensation, mood, and immune function (Tibiriça, 2010; Cani, 2012). These discoveries, insignificant though they may have seemed, have highlighted a novel therapeutic approach towards the treatment of cancers (Flygare and Sander, 2008).

Research over the last two decades has strengthened this argument, provided compelling evidence that fatty acid amides, among others, are signalling elements of the ECS. Two of the key endocannabinoids initially elucidated are anandamide (AEA) and 2-arachidonoylglycerol (2-AG), these endogenous molecules mediate the actions of cannabinoid receptors throughout the body (Howlett and Mukhopadhyay, 2000). AEA is a high affinity partial agonist for CB_1, with weak CB_2 affinity, whereas 2-AG acts as a full agonist of both CB_1 and CB_2 with moderate affinity (Di Marzo and De Petrocellis, 2012; Pertwee, 2015).

Other notable endocannabinoids include; oleoylethanolamide(OEA), palmitoylethanolamide (PEA), steaoryl ethanolamide(SEA), N-arachidonoyl-dopamine (NADA), virodhamine(VD), 2-arachidonyl glycerylether(noladin ether)

and oleamide (OA)(Sagar *et al.*, 2009; Ezzili, Otrubova and Boger, 2010). Endocannabinoids are synthesized on demand by a collection of enzymes, AEA is catalysed from N-acyl-phosphatidylethanolamine (NAPE) by NAPE-specific phospholipase D(NAPE-PLD) (Pacher *et al*., 2006). 2 –AG is produced from diaglycerol (DAG) by either DAG lipase(DAGL) α or β, though most is generated by the DAGLα (Di Marzo and De Petrocellis, 2012; Murataeva, Straiker and Mackie, 2014). Once their function has been achieved, AEA and 2-AG are degraded by fatty acid amide hydrolase(FAAH) and monoacylglycerol lipase(MAGL) respectively (McKinney and Cravatt, 2005; Mouslech and Valla, 2009; Fu *et al.*, 2012; Zou and Kumar, 2018). The use of inhibitors to block endocannabinoid degradation enzymes *in-vivo*, provoked increased tumour concentration of both AEA and 2-AG in tumours and resulted in a decrease in tumour growth (Bifulco *et al.*, 2004). The levels of both of these endocannabinoids are increased by up to three-fold in ex-vivo CRC tissue, suggesting these molecules may possibly be a physiological response to increased proliferation (Ligresti *et al.*, 2003).

Cannabinoid receptor 1(CB_1) is a G-protein coupled receptor that is found primarily in the central nervous system, but also been identified throughout all peripheral tissues (Pacher *et al*., 2006; Schaich *et al.*, 2014). The type 2 cannabinoid receptor (CB_2), is 40% homologous in structure to CB_1 but is instead dispersed throughout the peripheral nervous system, and are expressed predominantly in immune cells as well as throughout the brain, and are emerging as integral mediators of immune function and inflammation (Massa *et al*., 2005; Mackie, 2006). In addition to the widely known cannabinoid receptors, CB_1 and CB_2, the endocannabinoid system has multiple other receptors that contribute to its

function; Transient receptor potential vanilloid channels (TRPV's), G protein-coupled receptor's (GPR's), 5 hydroxytryptamine receptor 1A (5-HT_{1A}) and peroxisome proliferator-activated receptors (PPAR's), though it is accepted that there are yet more to be discovered (Ligresti *et al.*, 2006; Pistis and Melis, 2010; Battista *et al.*, 2012; Di Marzo and Piscitelli, 2015; Sharkey and Wiley, 2016). Studies exploring the underlying signalling mechanisms of the ECS have proposed these receptor families as mediators of key cellular responses, including those involved in cancer (Flygare and Sander, 2008; Pyszniak, Tabarkiewicz and Łuszczki, 2016). All elements of the ECS have been detected in CRC tissues, the levels of these elements are augmented in CRC adenomas compared to healthy tissue (Ligresti *et al.*, 2003).

Our developing knowledge of cannabinoid signalling is likely to further expand the therapeutic possibilities of manipulating the ECS (Marzo *et al*., 2004). This exploration has already identified this as a tool for the management of colorectal diseases (Pacher *et al*., 2006; Di Marzo and De Petrocellis, 2012). Based the existing evidence reporting the implication of these receptors in various cancer pathology and progression we aimed to refine this knowledge and explore the mediatory role these receptors may play in CRC.

1.6.1 The ECS and the GI Tract

The ECS and its components have been detected throughout the gastrointestinal tract (Wright *et al.,* 2008). The ECS is a modulator of the gastrointestinal system, regulating gastric motility, secretion, emptying and permeability (Pertwee, 2001; Alhamoruni *et al.*, 2012). In addition, the ECS regulates gut homeostasis, including gastrointestinal motility, visceral sensation and inflammation, but most recently in the pathogenesis of inflammatory bowel disease (Alhouayek and Muccioli, 2012; Acharya *et al.*, 2017). Endocannabinoid signalling has been shown to mediate the regulation and prevention of inflammation within colonic tissues by both reducing smooth muscle irritation and influencing inflammatory pathways (Massa *et al*., 2005; Marquéz *et al.*, 2009). Recent data from animal and human models has suggested that the ECS becomes overstimulated during inflammatory states (Di Marzo and Izzo, 2006; Marquéz *et al*., 2009). Studies have demonstrated that activation of both CB_1 and CB_2 can decrease the heightened intestinal motility and secretion elicited by inflammatory stimuli (Alhamoruni *et al.*, 2012; Izzo *et al.*, 2015). Both receptors are located within the cell membranes of mast cells, with CB_1 reducing the secretory response and CB_2 contributing to the regulation of cellular kinase pathways (Samson *et al.*, 2003). Signalling between the gut and the brain is also regulated by the ECS, emesis and vagal gut motor function are regulated by endocannabinoids in the brainstem acting on CB_1, CB_2 and (TRPV)-1 receptors (Storr and Sharkey, 2007). The activation of CB_1 receptors by endocannabinoids induces relaxation of the lower oesophageal, inhibiting gastric acid secretions and intestinal motility (Di Marzo and Izzo, 2006). Endocannabinoid signalling via the

CB_2 receptor has also been suggested to reduce colitis associated inflammation (Marquéz *et al*>, 2009). Several other receptors have been identified as contributing to the function of the ECS in regular gut physiology. The transient receptor potential vanilloid 1($TRPV_1$), peroxisome proliferator-activated receptors (PPAR's) and G protein-coupled receptor 55(GPR_{55}) play an integral role mediating in inflammation and permeability in the gut (Karwad *et al.*, 2016). The GPR-55 receptor is present throughout the rat intestines, and is upregulated when treated with an inflammation inducing lipopolysaccharide (LPS). Cannabinoids such as CBD appear to inhibit GPR_{55} activity and correct induced motility disturbances associated with LPS treatment (Lin *et al.*, 2011). There is mounting evidence to suggest that GPR_{55} is also activated by AEA and 2-AG (Kapur *et al.*, 2009; Sharir *et al.*, 2012). TRPV's are a sub family are a six member family of TRPV ion channels divided into two subtypes, $TRPV_1$ to $TRPV_4$ and $TRPV_5$/$TRPV_6$ (Julius *et al.*, 1997). AEA is a full agonist of $TRPV_1$ and is linked to pain (Di Marzo and De Petrocellis, 2012). $TRPV_1$ immunoreactive nerve fibres have been found to be increased 3.5-fold in colonic biopsies from IBS patients, suggesting a link to visceral hypersensitivity and pain in IBS (Akbar *et al.*, 2008; Lee *et al.*, 2016). PPAR activation has been implicated in a multitude of physiological and pathological processes including, lipid metabolism, feeding, energy balance, cognitive functions, epilepsy, addiction and inflammation (Battista *et al.*, 2012). Under physiological conditions the serotonin receptor 5-HT_{1A}, is responsible for the orchestration of the peristaltic reflexes responsible for proper digestive transit (Bülbring *et al*., 1958; Gershon, 2013). Under Pathological conditions and with interactions with inflammatory mediators, 5-HT_{1A} may contribute to inappropriate secretomotor activity,

contributing to pain and nausea (Grundy, 2008). Studies have demonstrated the role of the endocannabinoid system in the maintenance of intestinal barrier integrity (Cani, 2012). Previous research in our lab has highlighted the permeability modulating role of CB_1 and the endogenous cannabinoids, AEA and 2-AG (Karwad *et al.*, 2017). Multiple other literature sources support the modulation of the ECS within GI tract, may serve as a therapeutic tool in the treatment of colonic inflammation and subsequent cancer development (Mackie, 2006; Mouslech and Valla, 2009; Pisanti *et al.*, 2013; Pertwee, 2015; Lee *et al.*, 2016). Recent research by our team has highlighted the anti-inflammatory potential of CBD in the acutely inflamed colon and the implication of the ECS in its modulatory role of intestinal permeability (Couch *et al.*, 2017a; Karwad *et al.*, 2017). These investigations were based on a systematic review and subsequent meta-analysis of 51 *in vitro* and *in vivo* studies examining the effects of colitis (Couch *et al.*, 2018). This review assessed abundant preclinical data and demonstrated that cannabinoid drugs have significant anti-inflammatory effects in the gut, concluding that there was a need for more clinical research. Based on this same principle, we aim to apply the existing *in vitro* and *in vivo* knowledge to an explant model of human CRC.

1.6.2 Therapeutic targeting of the ECS in cancer

Since its initial discovery, exciting progress and insight has been gained into the role of the ECS in cellular homeostasis and health. The widespread implication of the endocannabinoid system in inflammatory and homeostatic pathways has prompted investigation into the potential therapeutic use of the ECS in cancer treatment

(Bifulco and Di Marzo, 2002; Pyszniak *et al*., 2016). The potential of these treatments is demonstrated in the over expression of cannabinoid receptors which is reported in a variety of malignant cancer cell types (Pyszniak *et al*., 2016). These novel therapies have been explored in cell lines from both forms of Hodgkin's lymphoma (Haskó *et al.*, 2014). Members of the GPR family, primarily, GPR_{55}, $_{119}$ and $_{18}$, and CB_2 were found in HCMEC/D3 human brain endothelial cells and A2058 melanoma cells, these findings were then utilised to demonstrate that activation of CB_2 reduced the adhesion of melanoma cells to brain endothelial cells, highlighting a potential preventative anti-metastatic target (Haskó *et al.*, 2014). This same study highlighted the strong overexpression of GPR55 in Hodgkin's lymphoma and non-Hodgkin's lymphoma cell lines (Haskó *et al.*, 2014). In these melanoma cell lines, CB_1, GPR_{55}, $TRPV_1$ receptors and COX-2 were highly expressed (Adinolfi *et al.*, 2013). In human MCF-7 breast cancer cells, both agonism and antagonism of $TRPV_1$ channels have a significant inhibitory effect on cell growth (Vercelli *et al.*, 2014). $TRPV_1$ activation by AEA provokes apoptosis by increasing intracellular levels of hydrogen peroxide (H2O2), and the release of cytochrome-C from mitochondria (Maccarrone *et al.*, 2000). Human glioma studies have highlighted elevated CB_1 and CB_2 expression alongside a decrease in the activity of NAPE-PLD, FAAH and MAGL (Wu *et al.*, 2012). Elevated levels of both CB_1 and CB_2 in glioma tissue were positively correlated to tumour malignancy (Pyszniak *et al*., 2016). The activation of these overexpressed CB_1 and CB_2 receptors has been shown to trigger the ceramide-ERK signalling pathway to promote apoptosis (Kogan, 2005; Sarfaraz *et al.*, 2006). Apoptosis can be additionally triggered by activation of either CB_1 or CB_2 which inhibits adenylyl cyclase activity, lowering both cyclic adenosine

monophosphate (cAMP) levels and protein kinase A (PKA) activity, by regulation of gene transcription enzymes (Guzmán, 2003; Kogan, 2005; Sarfaraz *et al.*, 2006).

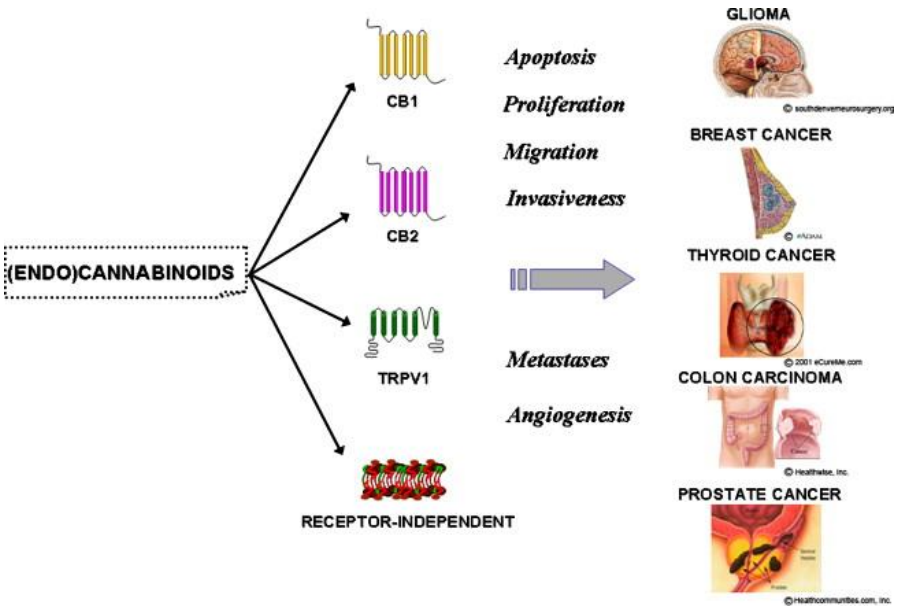


Figure 4. The physiological effects of cannabinoid activation of CB_1, CB_2 and $TRPV_1$ in various cancers (taken from Pisanti, 2009).

Targeting of the synthesizing and degrading enzymes of the endocannabinoid system has been proposed as potential endogenous means of tumour control (Flygare and Sander, 2008). Efforts have been made to block FAAH and MAGL enzymes as a means of increasing local endocannabinoid concentrations. Anti-tumour effects have been achieved by use of this method in thyroid, brain and prostate cancer (Bifulco and Di Marzo, 2002; Nithipatikom *et al.*, 2005; De Lago *et al.*, 2006; Endsley *et al.*, 2007). Though these therapies can be effective, blockading of endocannabinoid metabolism has been shown to have un-expected effects. This was observed in a study by Matas *et al*, whereby the presence of a FAAH inhibitor induced apoptosis not by increasing AEA levels but by preventing generation of its precursor ethanolamine (EA)(Matas *et al.*, 2007).

1.6.3 Targeting the ECS in CRC

Research exploring the physiological effects of CRC on the expression of various elements of the endocannabinoid system has highlighted a variety of potential therapeutic targets in the disease. This paper aims to apply the existing mass of ECS knowledge and apply it to a human explant model of CRC. For example, manipulating cannabinoid receptors with certain agonists can offer exciting insight into their clinical use in colorectal cancer management (Ellis *et al.*, 2000; Bendardaf *et al.*, 2008; Di Marzo, 2009). Many of the ECS receptors are known to be irregularly expressed, which continues to redevelop often resulting in the over expression of numerous receptors. In CRC, CB_2 over expression has been considered a poor prognostic factor and is associated with advanced lymph node involvement and vascular invasion (Martínez-Martínez *et al.*, 2015). Proto *et al.* highlighted that CB_1 receptor expression affects the response of cancer cells to steroidal growth hormones in DLD1 and SW620 cells (Proto *et al.*, 2012). These over expressed receptors are commonly present in cancer and offer an exciting target in the treatment of CRC. This has been demonstrated by CB_2 activation with synthetic agonists promoted CRC progression indicating this receptor may have a significant role in the survival and progression of CRC cells.

Such irregularities are also observed across the other receptors of the ECS suggesting these faults are contributing to cancer progression and metastasis. In HCT116 colon cancer cells, the GPR_{55} receptor was implicated in the migratory behaviour of cancer cells and may serve as a target in the prevention of metastasis (Kargl *et al.*, 2016). Similarly, the endocannabinoids themselves may be expressed

irregularly. AEA and its metabolite arachidonic acid are markedly upregulated in CRC biopsy tissues, when compared to adjacent healthy mucosa, this upregulation is even greater in cancer with lymphatic metastasis (Chen *et al.*, 2015). In a study conducted in 2003 by Ligresti *et al.*, tissue biopsies were collected during colonoscopy and relative levels of each endocannabinoid, their receptors, and enzyme expression were determined, and a CaCo-2 cell line used to test anti-proliferative effects. Tissue analysis confirmed the presence of AEA, 2-AG, CBRS and FAAH in all cells, the levels of endocannabinoids were up to three and two times larger in adenomas and tumours than normal mucosa. Use of CBR agonist, SR141716A, AEA and 2-AG, potently inhibited CaCo-2 cell proliferation via the CB_1 receptor (Ligresti *et al.*, 2003). Similarly, the endocannabinoid PEA is able to exert anti-proliferative effects by down regulating VEGF in C`aco-2 human colorectal carcinoma cell line but in a PPAR-α dependent manner (Sarnelli *et al.*, 2016). Pharmaceutically adapted cannabinoid compounds have shown significant benefit in disorders of the gut. An atypical cannabinoid O-1602, has been used as an anti-cancer modulator of the ECS, reducing the incidence of colitis by 30% and a 50% reduction in tumour area (Kargl *et al.*, 2013). Other cannabinoid receptor agonists such as the synthetic cannabinoid WIN55, 212-2, have been used to inhibit cancer cell survival by causing CB_1 mediated apoptosis (Xian *et al.*, 2009). Several other cannabinoid receptor agonists exhibiting pro-apoptotic and antineoplastic effects in SW837 rectal cancer cells (Gómez *et al.*, 2018). Similar anti-proliferative and cytotoxic effects have been produced in CaCo-2 cells using the synthetic cannabinoid CB HU 210, AEA, arachidonic acid and eicosapentaenoic acid, however the mechanisms of these effects were not mediated by either cannabinoid receptor

(Gustafsson *et al.*, 2009). Multiple reviews of this literature have concluded that manipulation of the ECS with cannabinoids is a tremendously promising anti-cancer therapy for colorectal cancer (Di Marzo, 2009; Solinas, Cinquina and Parolaro, 2015; Pisanti *et al.*, 2017; Uranga, Vera and Abalo, 2018).

1.7 Phytocannabinoids in CRC

One of the most widely researched and abundant phytocannabinoid is cannabidiol (CBD), which has emerged as one of the most promising cannabis derived anti-cancer molecules (Ligresti *et al.*, 2006; Pisanti *et al.*, 2017). Though both CBD and THC bind to the CB_1 receptor, CBD lacks the same psychoactive strength as THC, this is due to the allosteric nature of CBD's binding which in turn exerts differing physiological effects (Morales *et al.*, 2016). In fact, CBD is a non-competitive negative allosteric modulator of CB_1 receptors which may explain many of the *in vivo* effect derived by varying ratios of cannabinoids (Laprairie *et al.*, 2015).

This absence of psychoactivity coupled with its widely demonstrated anti-inflammatory properties, makes CBD of particular promise in cancer therapy (Ligresti *et al.*, 2006; Solinas, Cinquina and Parolaro, 2015; Petrosino *et al.*, 2018).

Cannabidiolic acid (CBDA) the acidic form of CBD which, likewise, lacks the psychoactivity of THC. CBDA has shown similar promise as an anti-inflammatory, having the ability to inhibit the release of pro-inflammatory COX-1 and COX-2 *in vitro* (Takeda *et al.*, 2008). As the medical understanding of the ECS and phytocannabinoids has matured, as too has our ability to apply this knowledge to a wider variety of pathologies. Several CBMP's already exist. These medicines are CBD

and THC based drugs, which are licensed and currently accessible through the NHS (Sativex, Nabilone). However, use is limited to rare cases of pain, spasticity (Russo, 2008; Russo *et al.*, 2016). These medicines have been well reviewed and are well tolerated by humans, demonstrating few adverse, or off-target effects (Lynch and Campbell, 2011; Johnson *et al.*, 2013; Bridgeman and Abazia, 2017). Crude animal research conducted in the 1970 on rhesus monkeys and dogs demonstrated the extreme levels of cannabinoid consumption needed to reach a lethal dose orally, with 9000mg/kg still being nonlethal (Thompson *et al.*, 1973). Considering the promising efficacy and safety of these novel and medieval phytocannabinoid based medicines, it stands to reason that we should continue to pursue the wider therapeutic possibilities of these medicines. By increasing our understanding of the ECS and its interactions with phytocannabinoids we can broaden the application of these treatments to diseases such as cancer. As yet, we remain decades behind in our understanding of the mechanisms and processes that underly the therapeutic effects of phytocannabinoids in humans (Hermanson and Marnett, 2011). Cannabidiol's interactions with the immune system and the expression of cannabinoid receptors throughout the gut, highlights the potential of cannabidiol a therapeutic in gut disorders (Fine and Rosenfeld, 2013). Already we have seen the oral administration of cannabidiol proving beneficial in the treatment of chronically inflamed states, with some research suggesting CBD as a strategic target for a new class of anti-IBD drugs (Costa *et al.*, 2007; Esposito *et al.*, 2013; Sarnelli *et al.*, 2016). CBD and CBDA, have proven to be beneficial in inflammatory disorders but little is still known about their anti-cancer effects in humans (Burstein, 2015). Multiple works have demonstrated the aforementioned overlap between sustained

inflammatory environments and carcinogenesis (Kraus and Arber, 2009; Triantafillidis, Nasioulas and Kosmidis, 2009; Grivennikov and Karin, 2011; Abrams, 2018). These widespread anti-inflammatory effects add further rationale to the use of CBD in cancer management. Questions raised about the safety, and efficacy of cannabidiol have been quelled by studies showing prolonged exposure to cannabinoids both in animals and humans noting an absence of adverse effects (Fine and Rosenfeld, 2013). Though there is a strong body of evidence supporting the reported anti-cancer effects of phytocannabinoids *in vitro* and *in vivo*, there is a great deal yet to be understood in human physiology (Śledziński *et al.*, 2018). In particular, our understanding of the exact mechanisms of these reported anti-cancer effects is still in its infancy (Ladin *et al.*, 2016; Velasco, Sánchez and Guzmán, 2016). Phytocannabinoids have long been proposed as anti-cancer agents and have demonstrated great therapeutic potential as future cancer treatment and management strategies (Patsos *et al.*, 2005; Hermanson and Marnett, 2011; Calvaruso *et al.*, 2012; Patil *et al.*, 2015; Blasco-Benito *et al.*, 2018). The anti-cancer effects of these phytocannabinoids are of particular promise in CRC, with studies observing a variety of promising cytotoxic and anti-proliferative effects (Patsos *et al.*, 2005; Aviello *et al.*, 2012a; Romano *et al.*, 2014a). CRC is currently lacking non-invasive treatment methods, with current UK clinical guidelines facilitating chemotherapy as a the only therapeutic option, which in some cases may be ineffective (NICE, 2011).

1.8 CBD and CBDA in Cancer

Cannabis derived compounds such as CBD are being becoming widely adopted by patients seeking to use these compounds to alleviate and manage their cancer symptoms but also too in a palliative capacity (Machado Rocha *et al.*, 2008; Waissengrin *et al.*, 2015; Pergam *et al.*, 2017). The significance of this cannot be understated. In a 2018 study of Canadian cancer patients, 43% of patients surveyed reported that they were currently in use of medical cannabis (Martell *et al.*, 2018). In this same year, a survey of Australian GP's demonstrated 61.5% of them had experienced one or more patient's enquiries about the general use of medical cannabis in the previous 3 months (Karanges *et al.*, 2018). Patients seeking benefit from cannabis medicines have typically been discouraged by the ineffectiveness of conventional treatments and widening attention to anecdotal reports (Webb and Webb, 2014b; Karanges *et al.*, 2018). The heavy influence of cannabinoids on the ECS and the gut coupled with its clear in-vitro anti-cancer efficacy, supports the use of these molecules in CRC. We propose the use of CBD and CBDA as a therapeutic molecules for treating CRC given their widely cited anti-inflammatory and anti-cancer effects in addition to the strong distribution of the ECS within the gut. In pursuit of more detailed information regarding the use of CBD and CBDA in CRC we performed a database search of MEDLINE, EMBASE and PUBMED for "((Cancer).ti,ab AND ((cannabidiol).ti,ab OR (cannabidiolic acid).ti,ab))" yielded 133 studies. Comparative studies, reviews, and animal studies were excluded, leaving primary research papers exploring the effects of CBD and/or CBDA in all human cancer cell lines. This exclusion criteria resulted in a collection of 21 publications citing anti-proliferative, anti-angiogenic, anti-metastatic and cytotoxicity of CBD and

CBDA various human cell lines (**Table 1**). Publications were analysed for the cell types explored, concentrations of CBD and CBDA investigated, the mechanisms identified, and the observed effect of these phytocannabinoids on the cancer cell line. All of the publications gathered, demonstrated beneficial outcomes when treated with CBD or CBDA, showing anti-cancer mechanisms in 9 different human cancer cell lineages varying from prostate to the brain (**Table 1**).

CBD **Study**	**Cell type**	**Concentration**	**Mechanism**	**Observed effect**
(Aviello et al., 2012)	CaCo-2 and HCT116	0.01–10 µM	CB_1, $TRPV_1$, $PPAR_\gamma$	↓proliferation
(Deng et al., 2017)	T98G, U251, and U87M glioblastoma cells	0.3–100 μM	Suspect GPR_{55}, CB_1, $TRPV_1$.	↓proliferation, ↓cell viability
(Fisher et al., 2016)	Neuroblastoma, SK-N-SH cells	0-50mg/mL	n/a	↓invasion, ↑ caspase 3 , ↓cell viability
(Fonseca, Correia-da-Silva and Teixeira, 2018)	Hec50co Endometrial cancer cell	0.01–25 µM	$TRPV_1$	↑LDH, ↑caspase −3/−7, ↑ROS
(Ivanov, Wu and Hei, 2017)	Glioblastoma multiform; U87MG (HTB-14), U118MG (HTB-15), and T98G (CRL-1690)	5–20 µM	CB_1	↑TNF/TNFR1, ↑TRAIL/TRAIL-R2 ↑JNK1/2, ↑ MAPK, ↑p38, ↓ERK1/2, ↓AKT1

(Ligresti et al., 2006)	MDA-MB-231 breast carcinoma	1-25 µM	$TRPV_1$	↑ROS, ↑ caspase 3
(Lukhele and Motadi, 2016)	HeLa, ME-180 and SiHa cervical cells	0-3.2 µg/ml	n/a	↑ caspase 3/7, ↓cell viability, ↑p53, ↓ATP
(McAllister et al., 2007)	MDA-MB231 and MDA-MB436	0.1- 1.5 µmol/L	n/a	↓Id-1, ↓invasion
(McAllister et al., 2011)	MDA-MB231 cells	1.5 µM	n/a	↑ERK, ↑ROS, ↓Id-1
(Morelli et al., 2014)	U266 multiple myeloma cell lines	20 µM	$TRPV_2$	↑ROS, ↓cell viability, ↓ERK, ↓AKT
(Ramer et al., 2010)	Cervical (HeLa, C33A) and lung (A549)	10 µM	CB_1, CB_2 and $TRPV_1$	↑VEGF
(Ramer et al., 2013)	Lung cancer primary tissue, and cell lines (A549, H460)	3 µmol/L	PPAR-γ	↑COX-2, ↑PPAR-γ
(Romano et al., 2014)	DLD-1 and HCT116 colorectal carcinoma cells	0.3 - 5µM	CB_1	↓proliferation
(Ruhaak et al., 2011)	HT29 human colon adenocarcinoma cell line	300µM	n/a	↑COX-2, ↓COX-1, ↑PG2
(Srivastava, Srivastava and Brouhard, 1998)	Eosinophilic leukemia (SRIH-B ATL), NK leukemia cell line (SRIK-NK)	2.5-10 µg/ml	n/a	↓IL-10, ↓IL-8 ↓MIP-1α,β
(Sultan, Marie and Sheweita, 2018)	T-47D MDA-MB-231 breast cancer cells	1–7 µM	PPARγ	↑PPARγ, ↓mTOR, ↓cyclin D1
(Kosgodage et al., 2018)	Prostate cancer (PC3), hepatocellular carcinoma	1 and 5 µM	n/a	↓STAT3, ↓EMV, ↓cell viability

	(HEPG2), breast (MDA-MB-231)			
(Yamada et al., 2010)	T24 and RT4 bladder cancer cells	1.5-60 µM	$TRPV_2$	↑Annexin-V, ↑intracellular Ca2+
CBDA Study	**Cell type**	**Concentration**	**Mechanism**	**Observed effect**
(Ruhaak et al., 2011)	HT29 human colon adenocarcinoma cell line 300µM	300µM	n/a	↓COX-1, ↓COX-2, ↑PGE2
(Takeda et al., 2012)	MDA-MB-231 breast cancer cells	5 - 25 µM	n/a	↓COX-2 ↑RhoA ↑PKA
(Takeda et al., 2014)	MDA-MB-231 breast cancer cells	25 µM	n/a	↑SHARP1, ↓Id-1, ↓COX-2
(Takeda *et al.*, 2017)	MDA-MB-231 breast cancer cells	1 to 50 µM	not GPR_{55}	↓c-fos

Table 1. A literature table outlining the existing studies that cite and support the efficacy of CBD and CBDA inhuman cancer cell lines.

This literature gathered supports the use of both CBD and CBDA as anti-cancer agents. CBD and CBDA have clearly demonstrated anti-proliferative, cytotoxic and anti-inflammatory effects within 22 studies and across multiple human cancer cell lines. Four of these studies have cited anti-tumorigenic effects in human colorectal cancer lines. In colorectal cancer cell lines these anti-tumorigenic effects include, reductions in the expression of inflammatory cytokines, capacity to modulate COX-1/2 expression, decreases overall cancer cell proliferation (Tsujii *et al*., 1997). Excitingly, CBDA has been shown to reduce the expression of both COX-1 and COX-2 in HT29 human colon adenocarcinoma cell line (Ruhaak et al., 2011). This Anti-COX activity was reproduced by Takeda et al., who demonstrated anti-COX-2 activity in MDA-MB-231 breast cancer cells (Takeda et al., 2012). Several studies have

highlighted the potential of CBD to increase the production of apoptotic markers such as caspases 3 and 7, reported in breast carcinoma, endometrial cancer and neuroblastoma (Ligresti *et al.*, 2006; Fisher *et al.*, 2016; Fonseca, Correia-da-Silva and Teixeira, 2018). Several receptors have also been identified as mechanisms contributing to these observed effects, including the much discussed cannabinoid receptors (CB_1 and CB_2) and the disputed $TRPV_1$ receptor (Aviello *et al.*, 2012a; Couch *et al.*, 2017a). This data provides a solid rationale for the use and further exploration of CBD and CBDA as anti-cancer agents. Clearly, manipulation of the ECS in cancer demonstrates remarkable selectivity for cancer cells, sparing normal and healthy tissue (Flygare and Sander, 2008). Such selectivity makes the ECS an exciting therapeutic target, which can be manipulated in a variety of methods to our benefit (Proto *et al.*, 2012; Pisanti *et al.*, 2013).

The handful of CBMP's already clinically approved for use in the NHS, have demonstrated safety and efficacy in the long term (Russo, 2008; Russo *et al.*, 2016). Despite the potential for offsite targets when utilising CBMP's, unwanted sequalae will be avoided by further exploration of the mechanism of endocannabinoid interactions with the body and how these are affected by cannabinoids (Calvaruso *et al.*, 2012). Roger Pertwee has proposed several methods to limit unwanted off target effects. It is strongly advised that (i) future strategies target cannabinoid receptors outside of the blood brain-barrier, (ii) target cannabinoid receptors specific to the target tissue, (iii) target upregulated cannabinoid receptors and selectively target CB_2 and/or adjunctive receptors (multi-targeting) (Pertwee, 2012). Based on the existing literature we believe that compounds that potentiate the endocannabinoid system rather than having specific receptor effects, this we

believe will have a more therapeutic effect rather than forcing systematic inhibitions of integral endocannabinoid regulation (Lambert and Fowler, 2005). Despite the rich distribution of research in animal models, little work has been done to demonstrate the effects observed in animal studies, in human tissue. As a result, we wish to provide further understanding of these effects and determine whether these already observed in-vitro effects can translate into effective clinical findings using human explant tissue from colorectal cancer patients. This research would be the first of its kind to explore the effects of CBD and CBDA in *ex-vivo* human tissue and their ability to potentiate the physiology of human tissue. Using the CaCo-2 cell model wish to demonstrate the efficacy of CBD and CBDA prior to the use of *ex vivo* tissue. The use of this cell line allows us to predict the effects of CBD and CBDA in patients but also offers support to any positive findings we do produce in human tissue. With just 21 publications exploring the widely acclaimed anti-cancer effects in human cell lines, and just one exploring these effects in human explant tissue, we highlight a significant gap in knowledge and a lack of clinical research in this field which warrants further and sustained exploration.

1.9 Summary and Aims

The therapeutic manipulation of the ECS offers significantly potential in the treatment of a wide variety of cancer types. A solid body of evidence exists to support the efficacy of cannabinoid compounds in human physiology (Kogan *et al.*, 2007; Guindon and Hohmann, 2011). Though the anti-cancer effects of CBD and CBDA are well recorded (Table 1) these actions are yet to be elucidated in CRC models (Kraus and Arber, 2009). We hypothesize that CBD will have anti-

proliferative and cytotoxic effects in explanted human CRC, and that these effects will be mimicked, in some way, by CBDA. We aim to advance human scientific understanding of the effects of ECS immunomodulation in the treatment human CRC. The aims of this experiment are therefore as follows;

1. To demonstrate the previously identified anti-proliferative, cytotoxic and anti-metastatic effects of CBD and CBDA in human cells and explant tissue.
2. Identify the mechanisms by which CBD and CBDA exert their anti-cancer effects *in vitro* and in *ex vivo* human colorectal cancer.
3. Explore the implication of the ECS in the potentiation of the physiological effects exerted by CBD and CBDA.

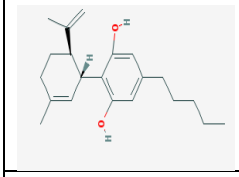
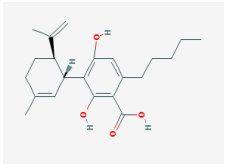
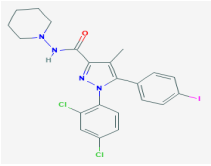
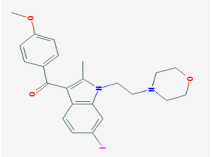
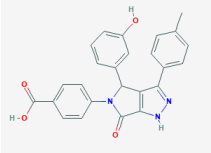
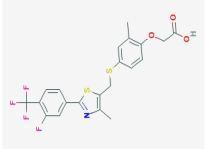
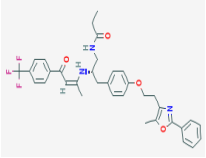
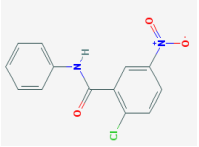
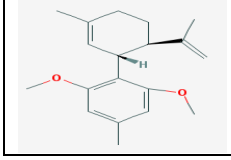
2 Methods

2.1 Chemicals, drugs and solutions

Tables **2** and **2.1** Lists outlining the drugs and solutions that were utilised at various stages of this study.

Chemical/Solution	Application	Protocol	Components
(MEME) cell culture medium	Culture of CaCo-2 cell line	10% FBS, 1% Penicillin and streptomycin , 1% Non-essential amino acids	Earle's salts, non-essential amino acids and sodium bicarbonate
Trypsin/EDTA (science cell, USA)	To detach cells from culture surfaces during passaging procedure	No additions	0.25% trypsin, 0.5mM EDTA, 1mM sodium pyruvate and 10mM HEPES
Phosphate buffered saline (PBS; Sigma-Aldrich, UK)	Resazurin diluent, cell/tissue washing	No additions	137mM NaCl, 2.7mM KCL, 10mM Na_2HPO_4, 1.8mM KH_2PO_4
Chemical/Solution	**Application**	**Protocol**	**Components**
RIPA buffer (Sigma-Aldrich, UK)	Collecting cells for protein quantification	Add 1 phosphatase and protease inhibitor tablets (Roche, Germany) per 10mL of buffer	50mM Tris-HCl, pH 8.0 with 150mM sodium chloride, 1%Igepal CA-630, 0.5% sodium deoxycholate and 0.1% sodium dodecyl sulfate
(MEME) Human tissue culture	Culture of Human tissue/ serum starving CaCo-2 cells	1% FBS, 1% Penicillin and streptomycin , 1% Non-essential amino acids	Earle's salts, non-essential amino acids and sodium bicarbonate

Table 2. A list of commonly used chemicals/solutions. BSA = bovine serum albumin; FBS = foetal bovine serum; MEME = minimum essential medium eagle; RIPA = radio immunoprecipitation assay; EDTA = ethylenediaminetetraacetic acid.

Name	Stock/solvent	Supplier
Cannabidiol (CBD) 	10mM, Ethanol,	Tocris, UK
Cannabidiolic Acid (CBDA) 	10mM, Acetonitrile (ACN)	Sigma Aldrich, UK
AM251 	10mM, DMSO	Tocris, UK
AM630 	10mM, DMSO	Tocris, UK
CID-16020046 	10mM, DMSO	Tocris, UK
GW0742 	10mM, DMSO	Tocris, UK
GW6471 	10mM, DMSO	Tocris, UK
GW9662 	10mM, DMSO	Tocris, UK
O-1918 	10mM, DMSO	Tocris, UK

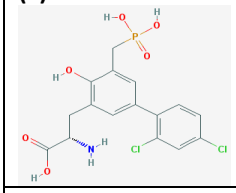
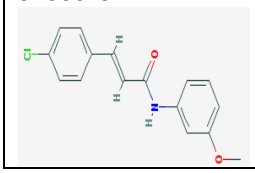
(S)-WAY-100135 	10mM, DMSO	Tocris, UK
SB366791 	10mM, DMSO	Tocris, UK

Table 2.1. A list of all drugs used in this project. Drugs were store at the indicated stock concentration (-20°C) and made to working concentration via serial dilutions on the day of experimentation.

In all experiments, vehicle control was made up of the same solvent the stock solution of drug had been dissolved in (as described in table 2.1). In circumstances where several test concentrations were used, the concentration of the vehicle was identical to that of the top concentration of drug. If a combination of drugs was used, relevant volumes of each solvent were added to the vehicle (ethanol and DMSO).

2.2 Caco-2 Cell Culture

2.2.1 Cell culture working environment

Heterogeneous human epithelial colorectal adenocarcinoma cells were sourced from the European Collection of Authenticated Cell Cultures (ECACC). A vial of 5 x 10^6/mL CaCo-2 was removed from liquid nitrogen and immediately thawed in a 37°C water bath. After 2 minutes of thawing, cells were transferred directly to a T75 flask containing 10mL of pre-warmed 10% FBS MEME media and placed in a CO_2 incubator at 37°C. As per the manufacturer's guidelines, medium was changed the following day and every 2 days thereafter, to maintain the proliferative potential of the cell line. The culturing of the CaCo-2 cell line was conducted under sterile conditions, using an advanced biological safety cabinet (Panasonic, USA). Prior to work conducted in the safety cabinet, airflow was stabilised, the working area within the cabinet was then sterilised using 70% IMS ethanol. All objects and materials used (pipettes, flasks, bottles etc.) were sterilised using 70% IMS ethanol to minimise risk of infection/contamination. Cells were cultured in a 37°C, 5% CO_2 environment sustained by a CO_2 incubator (Sanyo, Japan).

2.2.2 Cell passage procedure

To preserve the proliferative potential of the CaCo-2 cell line, flasks were passaged every 4 days, at approximately 70-80% confluency, which was confirmed using light microscopy. Media was aspirated and the flask contents washed with 5mL of pre-warmed PBS to remove any residual MEME media that could potentially inhibit the action of the trypsin/ethylenediaminetetraacetic acid (EDTA). After rinsing, the PBS

was aspirated and 2mL of trypsin/EDTA added to the flask, placed in the incubator for 1 minute, and observed under a light microscope to confirm cell detachment from the base of the flask. 10mL of 10% FBS MEME media was added to the flask to prevent further digestion of cells and to neutralise the trypsin/EDTA. The remaining cell suspension was then transferred to a 15mL falcon tube. The falcon tube containing the cell suspension was then spun at 180 G for 5 minutes at room temperature (Eppendorf, Germany). The supernatant was carefully aspirated and 10mL of 10% FBS MEME media added to the pellet and cells carefully resuspended using a disposable Pasteur pipette. An estimation of the cell number was made by pipetting 10µL of the cell suspension into a TC10 automated cell counter (BioRad, USA). 1 x 10^5 cells were added to a T75 flask following dilution of the suspension media, each T75 flask was topped up with 13mL of 10% FBS MEME media, and then returned to the incubator.

2.2.3 Cell freezing procedure

At approximately 70-80% confluence a T75 flask of CaCo-2 cells was passaged as described above (2.2.2). The cell pellet was then resuspended in the appropriate volume of MEME medium (5% DMSO) to achieve 1.0 x10^6 cell per mL. 1mL samples of suspended cells were aliquoted into cryovials that were placed in a Cryo^{TM} 1°C freezing container and stored in a freezer overnight at -80°C. Cells were cooled at a rate of -1°C per minute. The following day, these vials were transferred to liquid nitrogen for long term storage at -196°C.

2.3 Monoculture experiments

2.3.1 Resazurin reduction assay

The resazurin reduction assay is a widely used method of determining cell viability, which relies on the mitochondrial reduction of resazurin to resorufin in an NADH dependent manner (Borra *et al.*, 2009; Czekanska, 2011). The reduction of resazurin can be used to determine if compounds, such as CBD, have effects on cell proliferation or induce cytotoxicity (Riss *et al.*, 2004). For the purposes of this study, resazurin salt was used to quantify the effect of CBD and CBDA on proliferating and confluent cells. Resazurin was selected over several other mitochondrial staining assays due to its cost effectiveness and its repeatable format (Riss *et al.*, 2004). The addition of resazurin and its subsequent conversion to resorufin develops a pink fluorescence which is proportional to the number of viable cells present within each well. This fluorescent reduction is then quantified using a microplate fluorometer setup to detect at 560 nm excitation / 590 nm emission. A series of 1mL aliquots were produced containing a 1mM stock solution of resazurin sodium salt. 1mM stocks were produced by dissolving, 0.25117mg of resazurin sodium salt in 1mL of PBS. Before each experiment, a stock vial was brought to room temperature, avoiding direct light exposure, and diluted 1/10 in PBS. 20µL of this 1/10 solution was added to each well following drug application, producing an in-plate end concentration of 20µM.

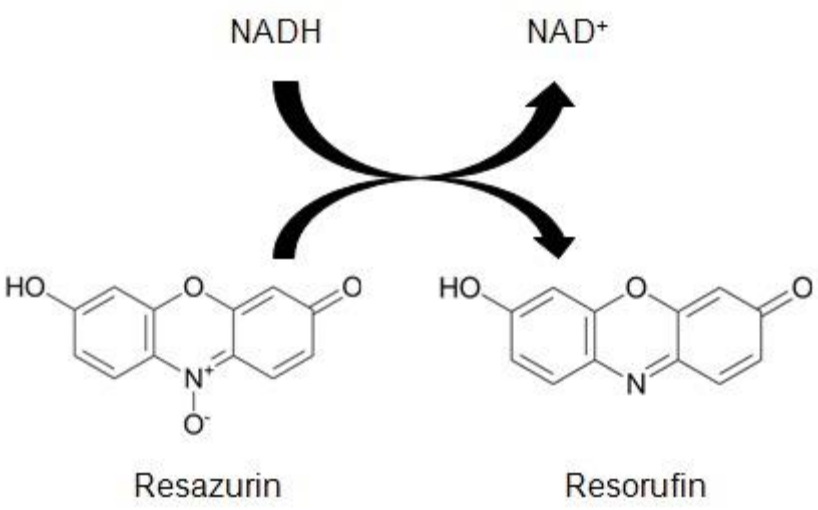


Figure 5. An image depicting the structure of resazurin and its reduction by NADH to its fluorescent product resorufin (Riss *et al.*, 2004).

2.3.2 Seeding density for proliferation assays

CaCo-2 cells were seeded at a variety of test densities to determine the optimal density for use with the resazurin sodium salt assay (**Figure 6**). CaCo-2 cells were seeded at densities ranging from 1000, to 200, 000 cells per well. The viability of these cells was observed over 72 hours and the growth of each cell group monitored using resazurin (see 2.3.1 for protocol). A seeding density of 10,000 cells per well was selected as colonies seeded at this density had the ability to sustain linear growth over the 72-hour period. The absorbance values provided by cells seeded at 10,000 cells per well provided a growth rate that fell within the centre of the absorbance range. The middle of the spectrum absorbance value allows for variations in the absorbance's detected following treatments with either vehicles or drug treatments to determine if CBD or CBDA offered growth inhibiting effects. This brief study highlighted the effect of varying seeding densities on CaCo-2 cell growth

over a 72 hour period, prior to the addition of any drugs.

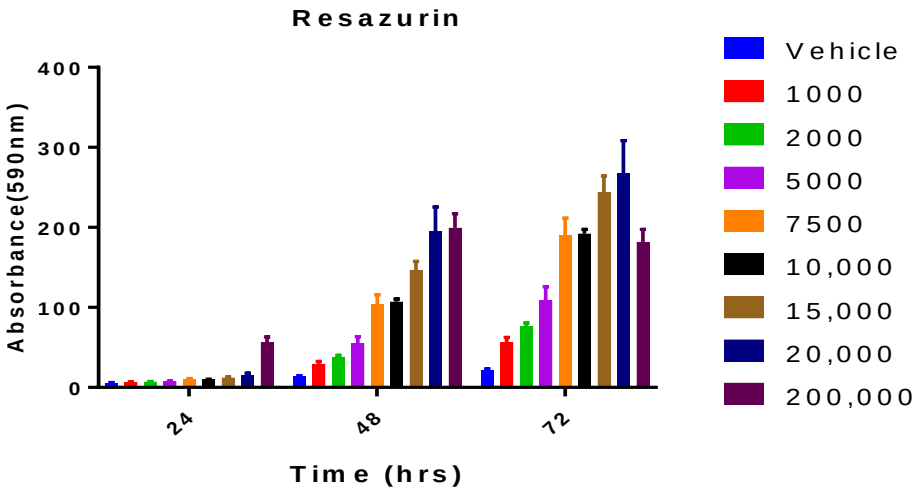


Figure 6. The effects of increasing seeding densities and their effects on culture growth as measured by resorufin production over a 72-hour period (**n=6**).

2.3.3 Proliferating cells studies

CaCo-2 cells were grown to 70-80% confluency in T75 flasks and serum starved for 24 hours allowing for growth arrest of cells prior to encourage a pronounced effect upon drug application. Cells were then harvested according to the procedure in 2.2.3 and seeded in a 96 well spectra-plate at 10,000 cells per well for 4 hours, allowing cells time to adhere to the bottom of the wells. Media was then aspirated. Wells were then treated with 100 µL of vehicle, CBD or CBDA at increasing concentrations of 1, 3, 10, 30, 50, 70 or 100µM in 10% FBS MEME. The effects of these drugs were monitored over a 72 hour period using the resazurin reduction assay (as described in 2.3.1).

2.3.4 Confluent cell studies

CaCo-2 cells were again serum starved and seeded at 10,000 cells per well as described in 2.2.2. Cells were incubated for 3 days and allowed to grow to confluency within the 96 well plate. 20 µL of resazurin was added at each time point and incubated for 4 hours (as described in 2.3.1). Following resazurin treatment, cells from each time point were imaged using a light microscope prior to collection. Follow the treatment period, cell media was collected from sample wells and placed into 2mL Eppendorf tubes and stored immediately at -80°C. In order to preserve protein condition, the following procedures were performed on ice, using chilled solutions to minimise protein degradation. A single tablet of protease and phosphatase inhibitors (Roche Applied Science, Germany), was dissolved in 10mL of radio immunoprecipitation assay (RIPA) buffer. Wells were washed with 100µL of PBS, aspirated, and then treated with 200µL of RIPA buffer. The plate was placed onto an agitator in the cold room for 30 minutes. Following agitation, cells were collected, placed into 1.5mL Eppendorf tubes, and spun at 10,000 RPM (1118G) in centrifuge maintained at 4°C. The supernatant was collected, and the pellet left undisturbed, both were then stored at -80°C.

2.3.5 Mechanisms of Action

In order to identify which receptors were implicated alongside CBD and CBDA in the anti-proliferative and cytotoxic studies, a series of antagonists were co-applied to CaCo-2 cells with CBD and CBDA at identified cytotoxic and anti-proliferative concentrations. Antagonists were applied to proliferating and confluent cells 20 minutes prior to drug administration. Using specific endocannabinoid receptor

antagonists, we aimed to inhibit the anti-cancer effects of CBD and CBDA, to demonstrate that CBD or CBDA were working through one of these antagonised receptors. By antagonising these receptors, we aimed to inhibit the therapeutic effect of CBD and CBDA, resulting in direct impairment of the anti-proliferative and cytotoxic effects. Inhibition of anti-proliferative and cytotoxic effects in the presence of a given antagonist corresponds to the involvement of this receptor in the exertion of CBD and CBDA's effects. The ability of each antagonists to inhibit the responses of CBD and CBDA were quantified using a resazurin reduction assay (outlined in 2.3.1). Antagonists were selected based on their efficacy in previous studies exploring the mechanisms of CBD and CBDA's effect. It is suspected that these molecules may have multiple sites of action and therefore in addition to the probing of CB_1 and CB_2, other putative receptors of the endocannabinoid system were also explored (Zuardi, 2008).

2.4 Human tissue

For this study we were able to utilise a human *ex vivo* model of CRC to provide a more clinically relevant means of testing (Novo *et al*., 2017). Patient Information and consent forms are provided (**Appendix 1 and 2).** All experiments and procedures received prior approval from the University of Nottingham Ethics Committee and local NHS Research Ethics Committee.

2.4.1 Patient recruitment and sample collection

Experiments that utilised human *ex vivo* colonic tumours were performed using tissue collected from patients undergoing elective laparoscopic colectomies for

colon cancer (**n=2**) at Royal Derby Teaching Hospitals NHS Trust, Derbyshire, UK. Consent was gained from patients on the morning of the procedure, in conjunction with the preoperative consultation with the attending surgeon. Immediately following excision, colon segments were transported from the surgical theatre to the pathology department, fresh, without formalin. Portions of tissue 2cm x 2cm were collected from the resected specimen. A sample was collected from both the tumour and the healthy mucosa, at least 10cm proximal to the tumour periphery. These segments were then transported in separate containers containing pre-chilled MEME supplemented with 1% FBS, 1% penicillin/streptomycin and 1% non-essential amino acids mixture (Sigma-Aldrich). The remaining explant tissue was retained by the attending pathologist to perform routine clinical analysis.

2.4.2 Tissue preparation and experimentation

Once in the laboratory, tissue samples were pinned on stylgard plates and washed with ice cold PBS. Sections of cancerous and healthy mucosa were dissected into 12, 3mm x 3mm sections and placed into 12 well plates. Tissue was treated in triplicate with 1 mL of 1% FBS MEME per well containing either a vehicle, or CBD or CBDA at concentrations of 30µm, 70µM or 100µM. This range of concentrations was selected based on data collected in earlier CaCo-2 studies, with the aim of generating a similar dose-dependent response. A reduced concentration of FBS was required to prevent over exposure of human tissue to external growth factors. Tissue was incubated at 37°C for 72 hours. At the end of this period, media was collected from each well and stored at -80°C for protein analysis. The remaining

tissue was washed with ice-cold PBS then dissolved in 300µL RIPA buffer and left on an oscillator in the cold room for 30 minutes. RIPA buffer and tissue were collected and placed into 1.5mL Eppendorf tubes. Samples were then mechanically homogenized in standard 1.5mL Eppendorf tubes using a polypropylene tissue "Squisher"™ (Zymo, EU). Tissue samples were place inside a temperature-controlled centrifuge and spun 10,000 RPM (1118G) for ten minutes, at 4°C. Supernatants were carefully collected, avoiding disruption of the tissue pellet, and placed in fresh Eppendorf's. Both the supernatants and tissue pellets were then placed in the freezer and stored at -80°C until further analysis.

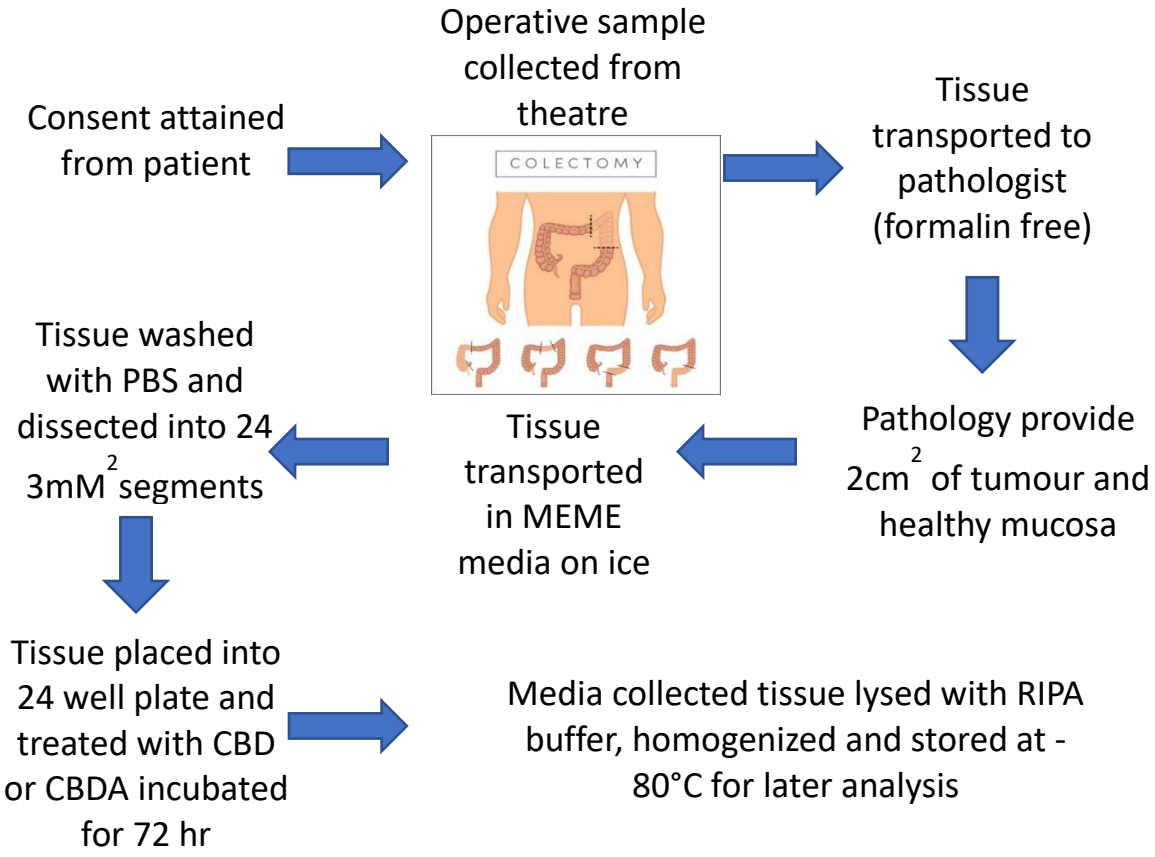


Figure 7. The experimental procedure for human tissue collection as depicted by a flowchart.

2.5 Biochemical and immunoassays

A variety of biochemical and immunoassays were performed on supernatants and media collected from the monoculture cells and human explant experimentation to examine tissue responses to drug treatments.

2.5.1 Total protein

A variety of assays are available for quantifying the total amount of protein present in a cell lysate. The analysis of total protein in a test sample is a key method of normalising tissue responses when tissue samples may vary in weight and size. It allows for the direct estimation of a cells productivity in the case of secreted proteins compared to the mass of tissue present, providing insight into the overall physiology of cells during a bioprocess (Reichelt *et al.*, 2016). In order to determine the effects of CBD and CBDA on total protein, we elected for the widely used Bicinchoninic acid (BCA) assay. Also known as the “Smith” assay after its inventor. The BCA assay was selected for its ease of use in the quantitation of total protein concentration compared to a standard (Reichelt *et al.*, 2016).

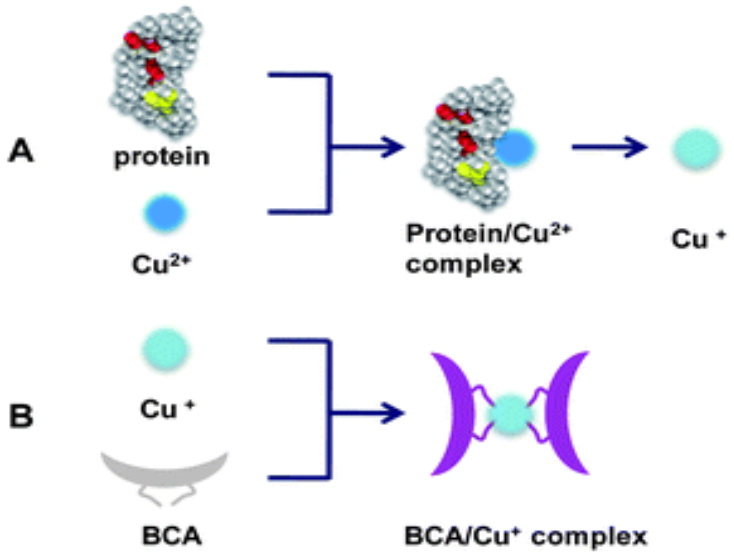


Figure 8. The generation of the fluorescent BCA/Cu^{+} complex following the reduction of Cu^{+} by the peptide bonds within the protein sequence (L. Chen *et al.*, 2012).

The BCA assay operates on the principle that the peptide bonds found in protein reduce Cu^{2+} ions from cupric sulphate to Cu^{+}, known as the *Biuret* reaction. This cuprous conversion is proportional to the amount of total protein present in the sample (Huang *et al.*, 2010). The colour development of this assay is generated by the chelation of a Cu^{+} ion by two BCA molecules forming a purple colour that absorbs light at a wavelength of 562nm (**figure 8**). The formation of the BCA/Cu^{+} complex is heavily influenced by the presence of several essential amino acids; cysteine, tyrosine, and tryptophan, commonly found in the amino acid sequence of all proteins.

Previously collected cell supernatants, stored at -80°C, were removed from the freezer and allowed to defrost on ice. A top standard was produced by dissolving 2 mg of Bovine Serum Albumin (BSA) in 1mL of RIPA buffer to generate a

top standard at a concentration of 2mg/mL, from which an 8 point standard curve was then produced. BCA working reagent was then made by mixing BCA solution with Copper (II) sulphate pentahydrate 4% solution (Sigma-Aldrich, UK) at a 50-1 ratio. Eppendorf's containing known standards were vortexed and 10µL of each added in duplicate to a 96-well plate. Each defrosted sample was then vortexed and 10µL of each sample added to the 96 well plate. Using a multichannel pipette, 100µL of BCA working reagent was added to each well, the plate was then incubated at 37°C for 30 minutes with a moderate agitation on a shaker plate. The plate was then read at 562nm using a multiscan spectrum plate reader with SkanIt Software 2.2 (Thermo, USA). Concentrations of total protein (µg/mL) were interpolated from the standard curve using Prism software (GraphPad, USA)

2.5.2 Cytotoxicity assay

A key focus of this study was to determine whether CBD and CBDA exerted necrotic or cytotoxic effects in CRC. The lactate dehydrogenase (LDH) assay was selected as it provides a cost effective means of assessing cell viability data whilst also offering a means of detecting toxicity in tissues (Smith *et al.*, 2011; Kaja *et al.*, 2015; Specian *et al.*, 2016). LDH allows for the quantification of cellular plasma membrane integrity, providing a mean for assessing the cytotoxicity of a drug (Chan *et al*., 2013). LDH quantification is of clinical significance, as serum levels of LDH isozymes are reflective of pathological conditions in certain tissues. Lactate dehydrogenase is a cytoplasmic oxidoreductase enzyme which when found extracellularly, indicates the presence of an impaired plasma membrane and thus compromise of cellular integrity, indicating necrosis (Vanderlinde, 1985). The LDH enzyme catalyses the conversion of pyruvate

to lactate with a concomitant inter conversion of NADH and NAD^+. This reacts with water soluble tetrazolium (INT) to yield a yellow coloured formazan product. The strength of this colour change directly correlates to the number of dead or damaged cells (Kumar *et al.*, 2018).

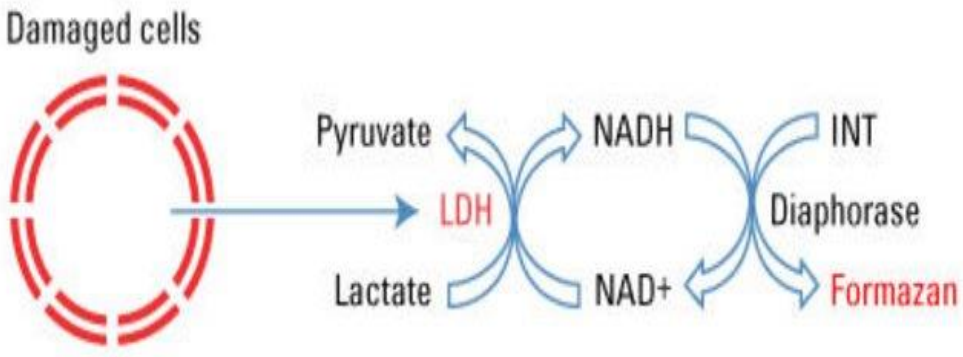


***Figure 9*.** The underlying mechanisms of coloured formazan formation from the presence of extra-cellular lactase dehydrogenase (LDH) (ThermoFisher, UK).

This assay was performed using media samples collected from confluent cells and human explant studies. Media samples, collected from previous experiments stored at -80 °C, were removed from the freezer and allowed to defrost on ice. Prior to assaying all testing reagents were reconstituted and brought to room temperature or kept on ice whilst in use, depending on reagent. A series of standards were produced with a top standard of 1.25mM NADH. 50µL of each standard was pipetted, in duplicate, into an optically clear 96-well plate. 4 wells were utilised for duplicates of a positive control (1µL per well) and two blank wells containing plain media (50µL per well). Samples were vortexed and 50µL added to a 96-well plate.

Reaction mix was prepared in a 50:1 ratio of assay buffer to substrate mix. 50µL was added to each well using a multichannel pipette bringing the final volume

of each wells to 100µL. Output was read immediately at an optical density of 450nm on a colorimetric plate reader. Readings were subsequently collected every minute following application of the reaction mix until the sample value were within the detection range. This procedure did not contain a standard curve. Data is expressed as a percent change from vehicle following removal of baselines. For the combination treatment study, LDH absorbance values were normalised to total protein content ([NADH]/mg/mL).

2.5.3 Enzyme linked immunosorbent assays (ELISA)

Enzyme Linked Immunosorbent Assays (ELISA's) are utilised to detect the presence of a ligand of interest within a test sample using antibody pairs. The cytokines targeted in this study, IL-6, IL-10, IL-17 and VEGF, have been strongly linked to the process of tumorigenesis and the maintenance of the tumour environment. To explore the use of CBD and CBDA as anti-cancer agents it was essential to investigate their ability to reduce the levels of several pro-carcinogenic cytokines in CRC. Existing literature has highlighted and increased presence of interleukins in CRC, which are understood to contribute to the tumour microenvironment (Kumari *et al.*, 2016). Through interleukin analysis we aimed to demonstrate that these heightened levels, could be attenuated with treatments of CBD and CBDA (Suarez-Cuervo *et al.*, 2003; Stanilov *et al.*, 2010; Wang *et al.*, 2014a). The concentrations of IL-6, 10 and 17 in samples collected from cancerous and healthy human explant tissue were compared using a Human cytokine respective DuoSet® ELISA kit (R&D Systems, US). Samples collected from each study were tested according to a

cytokine specific protocol outlined below. As is observed with interleukins, VEGF is similarly raised in CRC. VEGF is known to contribute to tumour CRC angiogenesis and maturation (Bendardaf *et al.*, 2008). With the same focus as in our interleukin assaying, we aimed to investigate the ability of CBD and CBDA to reduce the levels of VEGF in *ex vivo* CRC tissue. This test was performed with the aim of demonstrating that CBD or CBDA would be able to reduce the expression of VEGF in human colonic mucosa demonstrating possible anti-metastatic functions. To investigate this potential anti-angiogenic effect, a human VEGF-1 DuoSet® ELISA kit was utilised to analyse the expression of VEGF-1 in media collected from human tissue in response to CBD and CBDA treatments (R&D Systems, US). The following protocol was used for the ELISA's of these cytokines.

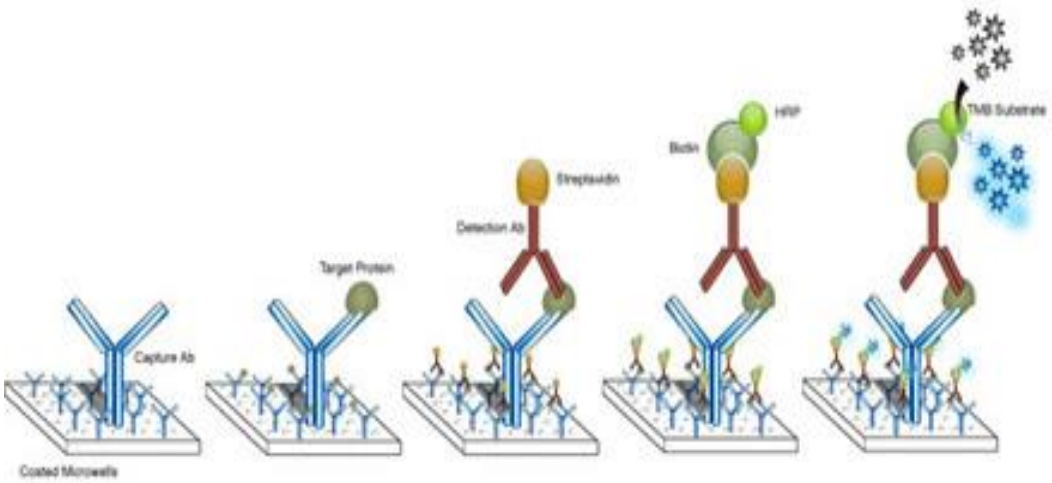


Figure 10. A visual representation of the typical procedural stages of a sandwich ELISA (*Sandwich ELISA schematic*, 2018)

A standard high-binding 96-well (Greiner, UK) is coated with an analyte specific capture anti-body and incubated overnight. The residual well content is washed from the wells using wash buffer (0.05% tween in PBS), and residual buffer

removed. This wash process is repeated three times between each step of the ELISA protocol. Residual liquid was removed, and wells then blocked for one hour with 200 µL of 1% BSA solution (reagent diluent), to reduce the likelihood of non-specific binding, and provide a more accurate reading. This blocking agent is later washed out as outlined above. The standards, quality controls, and media samples were then added to wells (100µL) and incubated for a minimum of two hours at room temperature to allow analytes to bind to the capture antibody. The wash procedure was repeated in preparation for the addition of conjugated streptavidin-horseradish peroxidase (HRP). Streptavidin-HRP, was diluted 1:40 in reagent diluent, and 100µL then added to each well. The plate was then covered with an adhesive cover strip and plate incubated at room temperature for 20 minutes, avoiding direct sunlight. The aspiration/wash procedure was then implemented again for a final time. Substrate solution was produced by mixing colour reagents *A* (H_2O_2) and *B* (tetramethylbenzidine (TMB)) in a 1:1 ratio. 100µL of substrate mixture was added to each well and incubated for 20 minutes avoiding direct sun light. This incubation period supports the generation of the soluble coloured product, the strength of which corresponds to the levels of analyte in each well. Once the colour reaches the desired intensity, 50µL of a standard stop solution (2N H_2SO_4) was added to wells. The optical density of the colour change was then determined using a microplate reader. Absorbance was measured at 450nm and at 570nm wavelength, values collected at 570nm were subtracted from 450nm data to correct for background interference. Although more efficient and less labour-intensive methods cytokine quantification are on offer, these methods were unfortunately beyond the budget of this research study.

2.6 Statistical Analysis

All data sets were processed using Prism 7 statistical analysis software (GraphPad, USA). All data points are expressed as means ± the standard error of the mean (SEM). Data sets collected over the 72-hour proliferation and confluent cell studies are expressed as a percent change from vehicle. Statistical significance was determined using two-way analysis of variance (ANOVA) due to the use of two independent variables, time (hours) and treatment (µM). Results were deemed statistically significant if $P < 0.05$. Outliers were identified and removed according to the Rout method (Q=1%). Dunnett's *post-hoc* testing was utilised as a means of multiple comparisons testing, to identify where significance variance from the control was present and confirm where these occurred. Dunnett's was utilised under the assumption that the data collected was normally distributed and possessed common variance (σ^2). P*ost-hoc* testing facilitated the identification of the CBD or CBDA concentrations that exhibited a significant response when compared to the control data. Data sets from our proliferation study that explored the mechanisms of action were analysed by way of two-way ANOVA, with Dunnett's *post-hoc* analysis. Data collected from studies exploring the cytotoxic mechanisms of CBD and CBDA at 48 hours analysed using one-way ANOVA due to agonist treatment being the sole variable in the study. Tukey's *post hoc* test was utilised to identify if and where statistical significance was present, and to compare the presence of significance changes between all means. In studies exploring the effect of combined antagonist treatment, one-way ANOVA was used with either Dunnett's post hoc test for proliferation studies, or Tukey's post hoc test for

confluent cell studies. Data collected from 72-hour studies exploring the impact of dual administration of CBD and CBDA on confluent cells were normalised to total protein content and analysed using one-way ANOVA with Tukey's *post hoc* test.

3 Results

3.1 Anti-Proliferation Studies

This study aimed to demonstrate the claimed anti-proliferative properties of CBD and demonstrate for the first time, the efficacy of CBDA as an anti-proliferative. This study explored the effect of increasing concentrations of both cannabinoids on the proliferation of CaCo-2 cancer cells over a 72-hour period. Under vehicle conditions, proliferating CaCo-2 cells demonstrated a linear increase in absorbance values(nm) over time, correlating to cellular proliferation and growth over the testing period. However, in the presence of CBD, this proliferation was strongly inhibited after 24h of treatment with 100 µM CBD ($P<0.0001$; ***Figure 11 A***). This same significant anti-proliferative effect was observed after 48 hours in the presence of 70µM ($P<0.005$) and 100µM CBD ($P<0.0001$). After 72-hour incubation, significant growth inhibition was achevied with 70µM ($P<0.05$) and 100µM CBD ($P<0.0001$). CBD exhibited promising dose dependent anti-proliferative effects at 24, 48 and 72-hour timepoints ***(Figure 11 A***). CBDA exhibited similar anti-proliferative potential to CBD, but seemingly with greater potency. Interestingly, CBDA demonstrated higher potency than CBD. Significant anti-proliferative properties were observed at concentrations as low as 30µM (***Figure 11 B***). The metabolism of resazurin to resorufin was inhibited by CBDA at lower concentrations than CBD, inhibiting cellular proliferation after 24hr at 50µM ($P < 0.01$), 70µM

(P<0.005), and after 48 hours at 30µM (P<0.005), 50µM (P<0.0001), 70µM (P<0.0001) and 100µM (P<0.0001). At the 72hr timepoint, CBDA inhibited proliferation at concentrations ranging from 30µM to 100µM (30µM p<0.005; 50, 70 and 100µM P<0.0001) ***(Figure 11 B***). Both CBD and CBDA demonstrated significant anti-proliferative properties at a variety of micro molar concentrations. This is the first time that CBDA has been shown to be a more potent anti-proliferative than CBD.

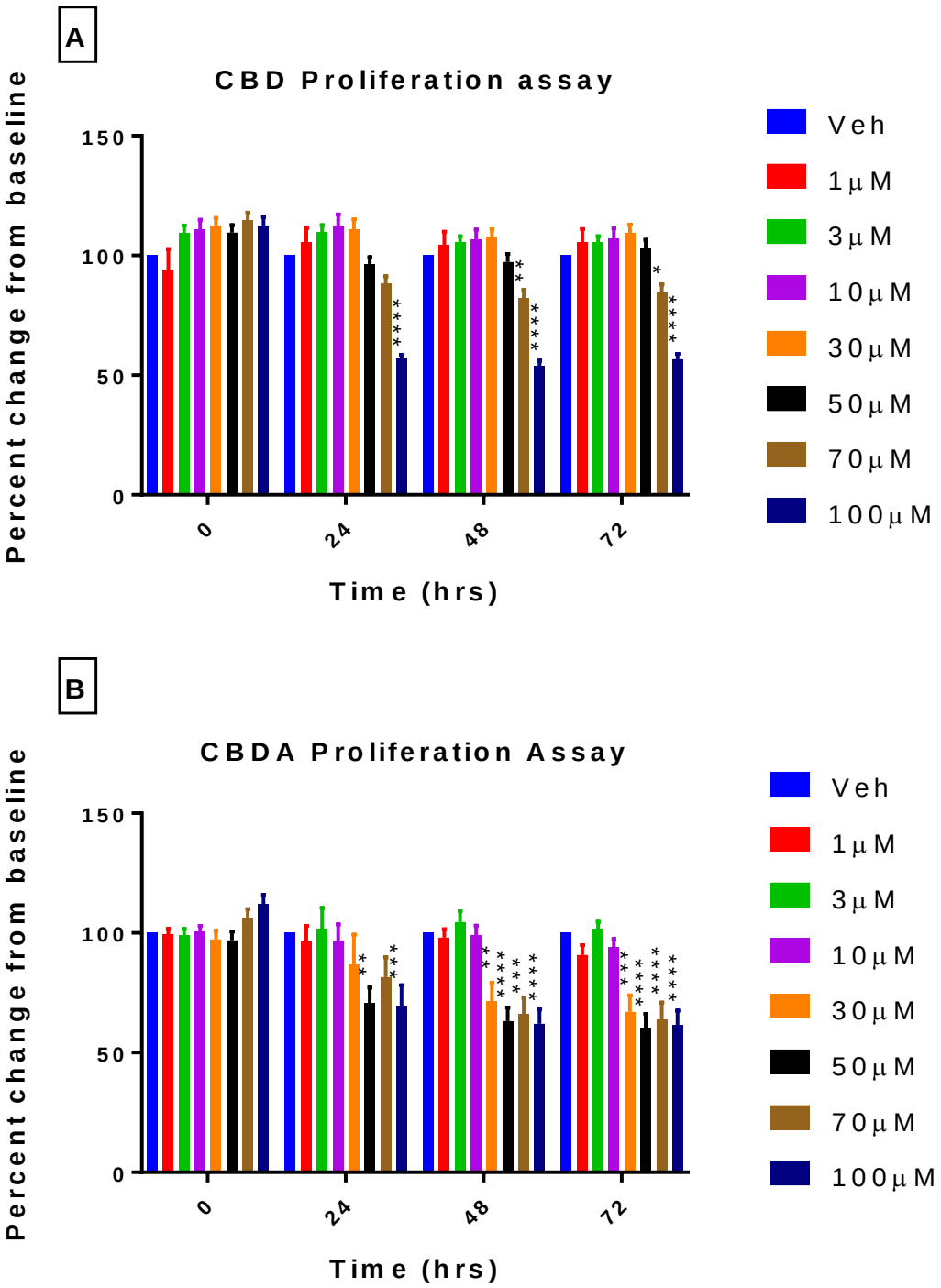


Figure 11. Effects of increasing micro molar concentrations of CBD (**A**; .**n=12**) and CBDA (**B**; **n=18**) on proliferating Caco-2 cells, observed using resazurin reduction assay. Data is expressed as mean ± SEM. Statistical analysis of increasing concentrations against vehicle conducted using two-way ANOVA, with Dunnett's *post hoc* testing; ****P<0.0001, ***P<0.001, **P<0.01,* P<0.05**).**

3.2 Cytotoxicity Studies

Confluent CaCo-2 cells under vehicle conditions exhibited a sustained degree of resazurin to resorufin conversion, demonstrating sustained activity and metabolism over 72 hours. This metabolic activity was inhibited by single applications of CBD at concentrations of at 50, 70 and 100µM which became more pronounced over time (***Figure 12 A***). CBD exhibited cytotoxic effects after 24 hours at concentrations of 70µM ($P<0.0001$) and 100µM ($P<0.0001$). This same cytotoxic effect was observed at 48hr with CBD treatments of 70µM ($P<0.0001$) and 100µM ($P<0.0001$). At 72hr, CBD exhibited cytotoxic effects at 50µM, 70µM ($P<0.0001$) and 100µM ($P<0.0001$). Images taken at the 72 hour time point demonstrate the reduction in cell number and health as the concentration of CBD is increased (***Figure 14 A***). Interestingly, initial treatment with 100 µM CBD at the 0hr time point triggered an increase in metabolic activity ($P<0.001$).

CBDA demonstrated lesser cytotoxic abilities than CBD, increasing metabolic activity from 100% to 180-220% after 24 hours incubation (***Figure 12 B***). No significant inhibition of metabolic activity was observed until the 72hour time point, at which 70µM CBDA significantly reduced the metabolic activity compared to the baseline ($P<0.01$).

Subsequent LDH analysis of the confluent cell media demonstrated that the decrease in resorufin production observed in the presence of CBD also resulted in an increase in lactate dehydrogenase from those same cells, supporting the theory that CBD has cytotoxic effects in CaCo-2 cells. These cytotoxic effects were apparent after 24hr of CBD exposure (70 and 100µM; $P<0.01$). This same degree of LDH release was also observed after 48hr (70µM $p<0.05$; 100µM $P<0.001$) and 72hr

(70µM $p<0.05$; 100µM $P<0.05$) (see ***Figure 13 A***). Though these results are significant, LDH release had not been normalised to protein content as cells were not collected until the end of the study. This resulted in larger variance between samples. In cells treated with 70 and 100µM CBD, secreted LDH was between 400 to 600% higher compared to those under vehicle conditions. The heightened presence of LDH extracellularly suggested a loss of plasma membrane integrity with necrotic effects, 24 hours post CBD treatment (Chan *et al*., 2013).

Increased LDH production was also observed in the presence of CBDA but to a far lesser extent than CBD. LDH analysis of confluent cell media from the CBDA study demonstrated significant cytotoxic potential at 48hr (70µM $p<0.05$) (***Figure 13 B***). Interestingly, we observed that the initial presence of CBDA at the time of the zero-hour reading exhibited reduced LHD release. This correlates with the observed increase in resorufin conversion observed in the confluent cells.

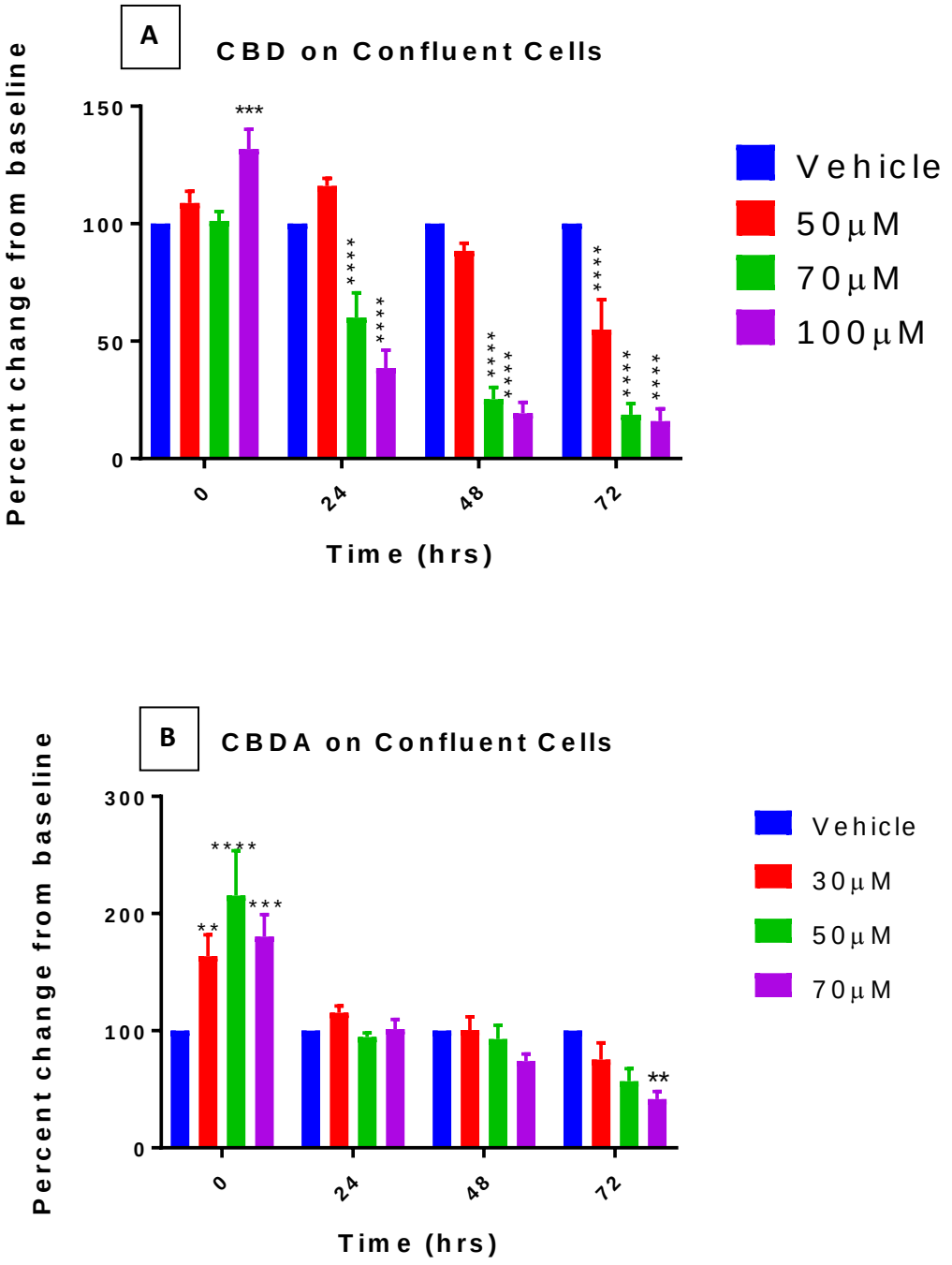


Figure 12. The ability of CBD (**A**) (50, 70 and 100 µM) CBDA (**B**)(30, 50 and 70µM) to effect resazurin conversion to resorufin in confluent Caco-2 cells over a 72 hour period. Data is expressed as mean percent change from baseline ± SEM. Statistical analysis of increasing CBD and CBDA concentrations against vehicle conducted using two-way ANOVA, with Dunnett's *post hoc* testing ****P<0.0001, ***P<0.001, **P<0.01(**n=9**)

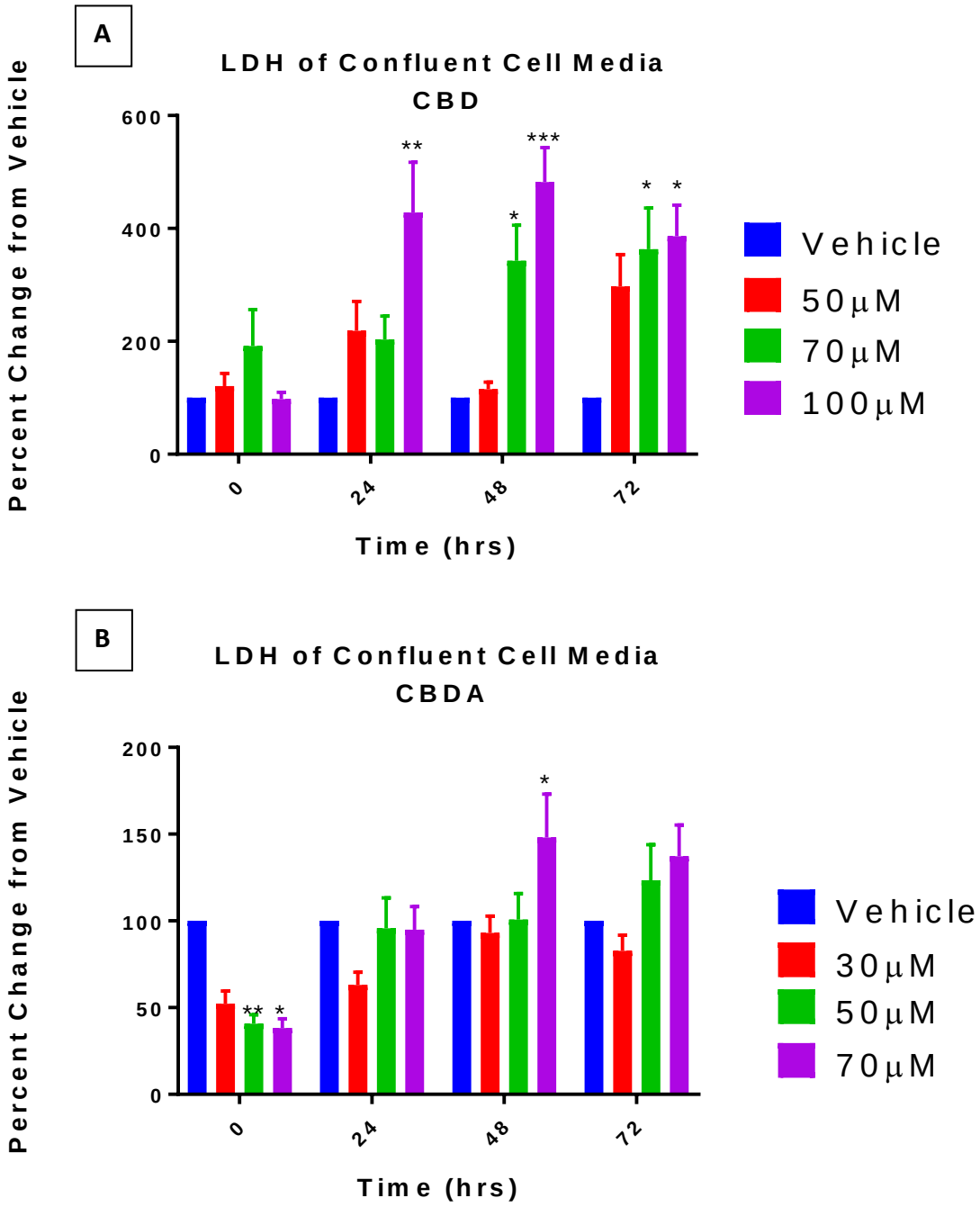


Figure 13. CBD application lead to increased extracellular lactate dehydrogenase, suggesting cytotoxic and necrotic effects of CBD in Caco-2 *in vitro* colorectal cancer model (**A**). Extracellular Lactate dehydrogenase levels in response to the application of CBDA 30, 50 and 70µM (**B**). Data are given as means with error bars representing ± SEM. Statistical analysis of increasing CBDA concentrations against vehicle conducted using two-way ANOVA, with Dunnett's *post hoc* testing. One outlier identified and removed using the Rout method (Q=1%); ***P<0.001, **P<0.01, * P<0.05.(**n=9**)

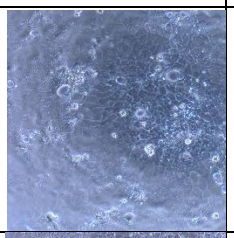
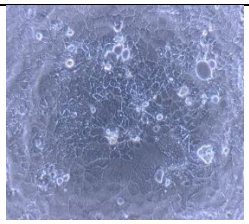
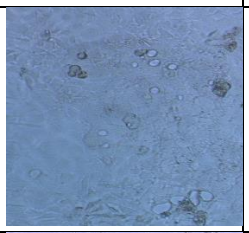
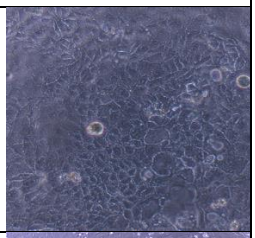
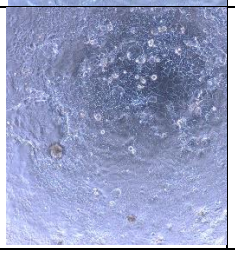
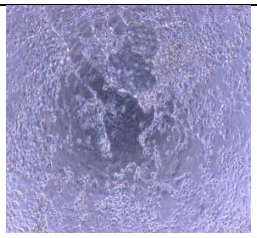
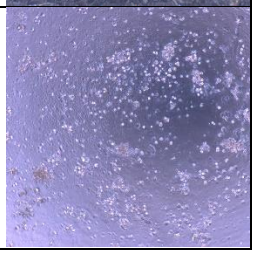
	Vehicle	50 µM CBD	70 µM CBD	100µM CBD
0 hr				
72 hr				

Figure 14 A. 20x magnification of representative confluent caco-2 cells treated with 50, 70 and 100µM CBD. Images were taken post resazurin treatment prior to plate reading at the 72hr timepoint.

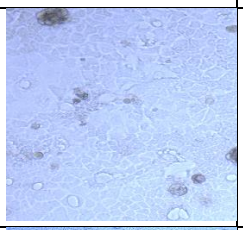
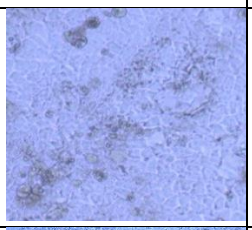
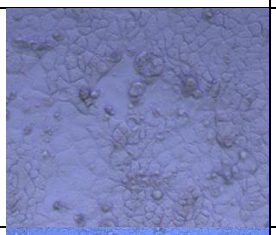
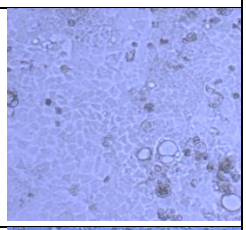
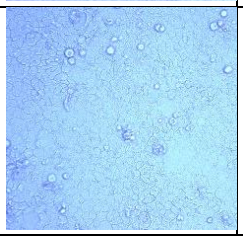
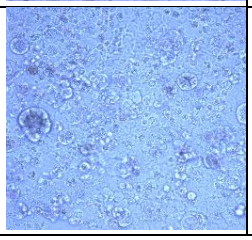
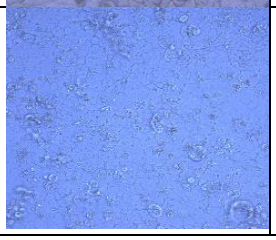
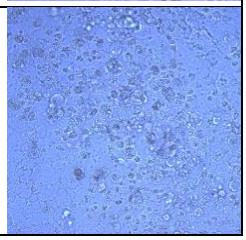
	Vehicle	30 µM CBDA	50 µM CBDA	70µM CBDA
0 hr				
72 hr				

Figure 14 B. 20x magnification of Confluent caco-2 cells after 72 hours incubation with 30, 50 and 70µM CBDA. Images were taken post resazurin treatment prior to plate reading at the 72hr timepoint.

3.3 Mechanisms of Action

The aim of this experiment was to investigate the possible receptors through which CBD and CBDA were exerting these anti-proliferative and cytotoxic effects.

Concentrations of 70µM CBD and 50µM CBDA were chosen based on their ability to exert significant anti-proliferative but still allow chance for inhibition of these effects by antagonism.

Antagonist name	Target receptor	Working concentration	Stock/Solvent	Supplier
AM 251	CB_1	100nM	10mM;DMSO	Tocris, UK
AM 630	CB_2	100nM	10mM;DMSO	Tocris, UK
GW 6471	$PPAR_\alpha$	500nM	10mM;DMSO	Tocris, UK
GW 9662	$PPAR_\gamma$	100nM	10mM;DMSO	Tocris, UK
O-1918	GPR $_{55/18}$	1µM	10mM;DMSO	Tocris, UK
(S)-WAY-100135	5-HT $_{1A}$	300nM	10mM;Purified water	Tocris, UK
SB 366	$TRPV_1$	500nM	10mM;DMSO	Tocris, UK
CID - 16020046	GPR $_{55}$	1µM	10mM;DMSO	Tocris, UK
GW 0742	PPAR $_\delta$	1µM	10mM;DMSO	Tocris, UK

Table 3. A table outlining the cannabinoid receptors targeted in the mechanism of action study, including their corresponding antagonists, working concentrations, solvent and supplier. 5-HT $_{1A}$ = 5-hydroxytyptamine receptor 1A; $CB_{1/2}$ = Cannabinoid Receptor 1/2, $GPR_{55/18}$ = G protein-coupled receptor 55/18; PPARα/γ/δ= Peroxisome activated receptor α/γ/δ; $TRPV_1$ = Transient receptor Potential vanilloid 1.

3.3.1 Anti-proliferation

In part one of this study, CBD exhibited significant anti-proliferative effects at 24, 48 and 72 hr (**Figure 11 A*).*** In this experiment, we aimed to inhibit these anti-proliferative effects with antagonist to isolate the functional pathways of our compound. As in part one, cells incubated under vehicle conditions continued to proliferate throughout the course of the study. This proliferation was once again inhibited by CBD at 24hr, 48hr and 72hr time points exhibiting a 50% reduction in proliferation compared to cells under vehicle conditions (**Figure 15 A**). The addition of antagonists had little inhibitory effect on CBD. The anti-proliferative effects of CBD were significantly supressed on two occasions, this occurred at 24 hours with pre-treatment of the $GPR_{55/18}$ receptor antagonist, 0-1918 (1µM; $P<0.05$), and the GPR_{55} antagonist CID (1µM; $P<0.01$). Despite the statistical significance of this inhibition, the antagonism was insufficient to return proliferation to basal levels. The presence of the remaining receptor antagonists did not curtail the anti-proliferative effects of 70µM CBD at any time point. CBDA exhibited a spectrum of effects throughout the 72 hours testing period. Comparable to the results observed in our initial proliferation studies (see **Figure 11 B**), CBDA treatment caused a significant increase in resazurin reduction at the time of treatment. Following the initial increase at the zero-hour time point, 50µM CBDA significantly inhibited proliferation compared to vehicle. The anti-proliferative effects of 50µM were partially inhibited by receptor antagonist O-1918 ($GPR_{55/18}$; 1µM), at 24 hours. (S)WAY (5-HT; 300nM), inhibited the effects of CBDA at 24 and 48 hours (***Figure 15 B***) SB366 ($TRPV_1$; 500nM) and CID (GPR_{55}; 1µM) significantly inhibited the anti-

proliferative effects of 50µM CBDA at the 24, 48 and 72hour time points (***Figure 15 B***).

3.3.2 Cytotoxicity

In the CBD study, cells under vehicle conditions demonstrated sustained metabolic potential which once again we were able to quell after 48hours using 70µM CBD ($P<0.0001$) (***Figure 16 A***). Following 30 minutes pre-treatment with individual receptor antagonists, CBD was applied with the aim of inhibiting the cytotoxic effect of 70µM CBD after 48 hours of incubation (as seen **in Figure 12 A**). Disappointingly, we were unable to inhibit the cytotoxic effects of 70 µM CBD with any of the receptor antagonists used (***Figure 16 A***). Interestingly, the potency of the drug was significantly augmented by the presence of receptor antagonists, AM630 (CB_2; 100nM; $P<0.01$), O-1918(GPR55/18; 1µM; $P<0.01$), (S) WAY (5-HT; 300nM; $P<0.05$) and CID (GPR55; 1µM; $P<0.05$) resulting in a further 5-10% decrease in cellular activity.

The cytotoxic effects of CBDA from part one of this study were once again demonstrated in confluent CaCo-2 cells (***Figure 17 B***). Confluent cell activity was once again significantly curtailed the over the 48hour trial period by a one-time application of 50µM CBDA ($P<0.001$). In the presence of receptor antagonists, we were able to inhibit the cytotoxicity of CBDA by pre-treatment of wells with several antagonists. The cytotoxic effect of CBDA confluent cells was inhibited by the presence of CID (GPR_{55};1µM) and (S) WAY(5-HT;300nM) at 48hours ($P<0.05$). Application of a PPARδ receptor antagonist GW0742 (1µM) also had a significant

inhibitory effect on CBDA's activity. Unlike CBD, CBDA's effects were inhibited by the antagonism of the GPR_{55}, 5-HT and PPARδ receptors.

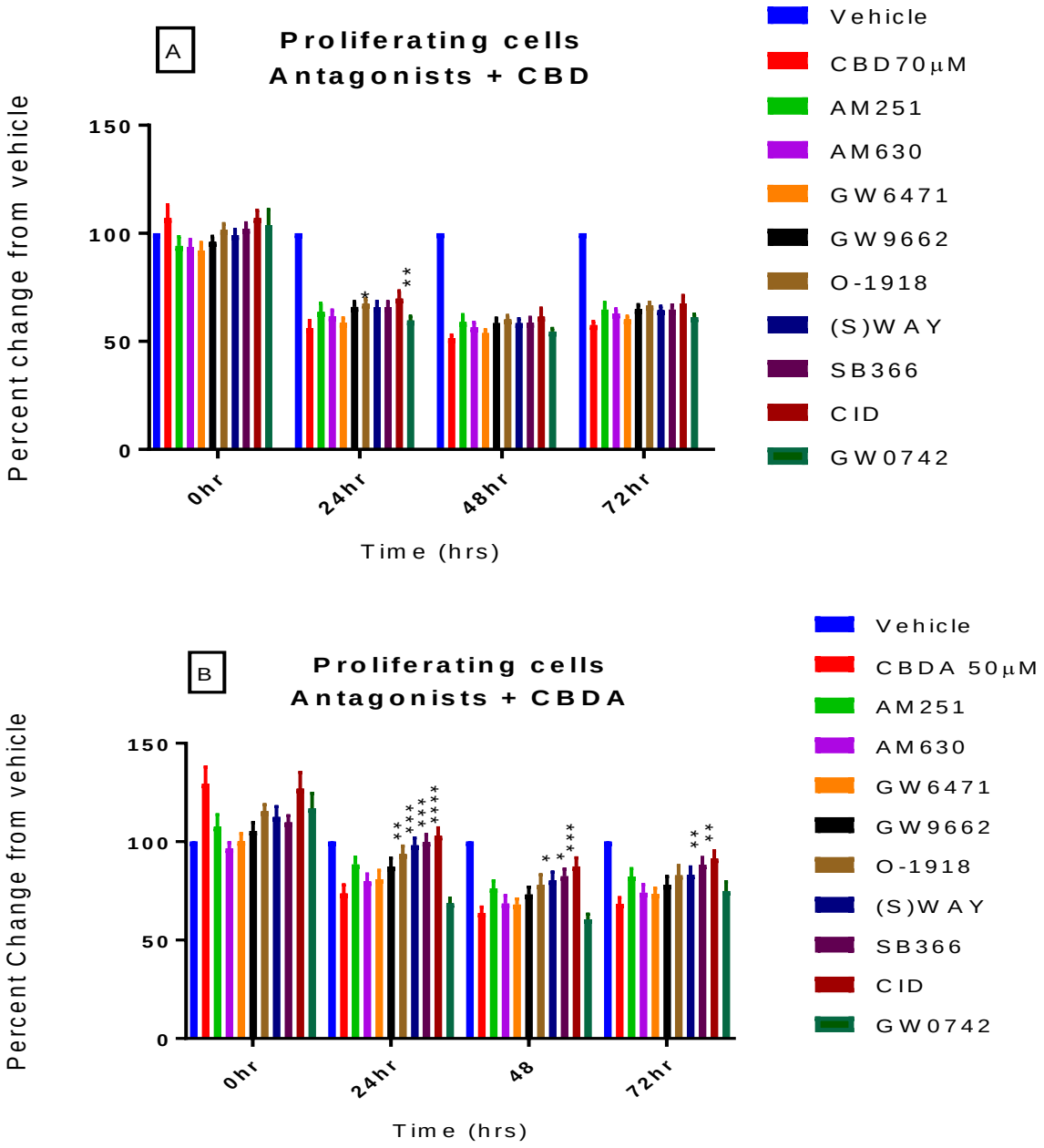


Figure 15. Inhibitory effects of various receptor antagonist on the anti-proliferative effects of 70 µM CBD (***A)*** and 50µM CBDA (***B***) on CaCo-2 cell over 72hrs in compared to vehicular conditions. AM251 (CB_1;100nM), AM630 (CB_2;100nM), GW6471 (PPARα;500nM), GW9662 (PPARγ;100nM),O-1918 (GPR55/18; 1µM), (S)WAY (5-HT;300nM), SB366 (TRPV1;500nM), CID (GBP55;1µM),GW0742 (PPARδ;1µM). Data are given as means with error bars representing ± SEM. Statistical analysis conducted using two-way ANOVA, with Dunnett's *post hoc* testing, (*) significance from drug treatment. ****P<0.0001, ***P<0.001, **P<0.01, * P<0.05.(**n=16**)

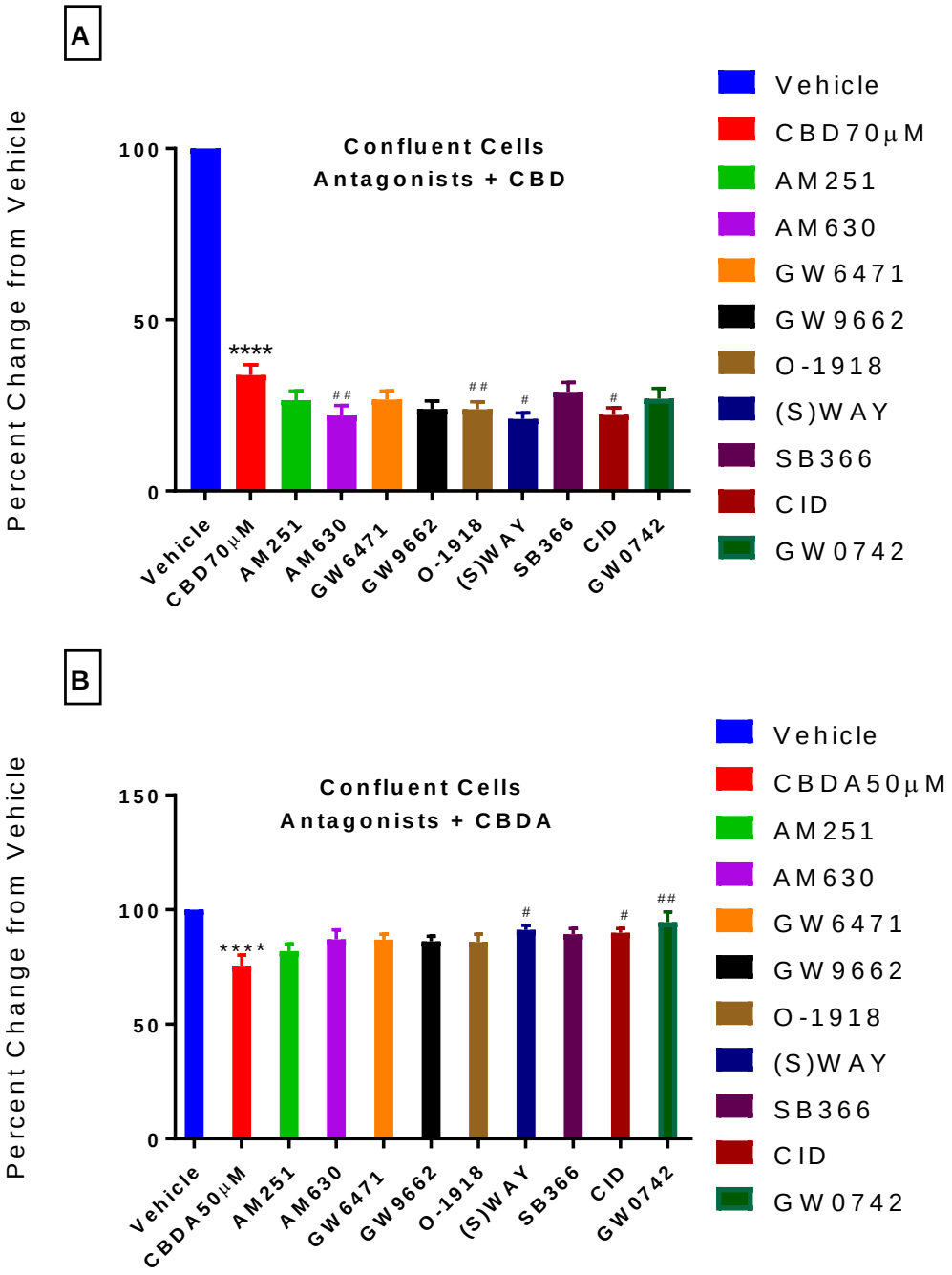


Figure 16. Effects of various receptor antagonists (AM251(CB_1;100nM), AM630(CB_2;100nM), GW6471(PPARα;500nM), GW9662(PPARγ;100nM),O-1918(GPR55/18; 1μM), (S)WAY (5-HT;300nM), SB366(TRPV1;500nM), CID (GBP55;1μM), GW0742(PPARδ;1μM)) on the cytotoxic effects of 70μM CBD(***A***) and 50μM CBDA (***B***) when applied to confluent cells for a 48 hour period. Data expressed ± SEM. Statistical analysis conducted using one-way ANOVA, with Tukey's *post hoc* testing; ****P<0.0001, ***P<0.001, **P<0.01,* P<0.05, (*) significance from vehicle, (#) significant change from drug (**n=16**).

3.4 Multiple Antagonist Treatments

Following the mechanism of action trials and the absence of a defined receptor or pathway, a final antagonist study was conducted investigating the effect a combination of all the receptor antagonists. This was done in an attempt to inhibit the cytotoxic and anti-proliferative effects of CBD and CBDA, which had appeared to be working non-specifically, through multiple interaction sites. As a result, if there are in fact multiple receptors are involved, it may require the use of multiple antagonists in order to inhibit the effects of CBD and CBDA.

Once again, when compared to cells under vehicle conditions, cells treated with CBD 70µM produced a significant anti-proliferative effect reducing proliferation by 60% from baseline (P<0.0001) (***Figure 17 A***). The effects of 70µM CBD were not inhibited by the presence of a combination of receptor antagonists, AM251 (CB_1;100nM), AM630 (CB_2;100nM), GW6471 (PPARα;500nM), GW9662 (PPARγ;100nM), O-1918 ($GPR_{55/18}$; 1µM), (S)WAY (5-HT;300nM), SB366 (TRPV1;500nM), CID (GBP55;1µM) and GW0742 (PPARδ;1µM). The same was observed for CBDA. CBDA treatment of 50µM had significant anti-proliferative effects(P<0.0001) (**F*igure 17 B***), with this effect also unhindered by the pre—treatment of cells with a combination of various receptor antagonists, AM251 (CB_1;100nM), AM630 (CB_2;100nM), GW6471 (PPARα;500nM), GW9662 (PPARγ;100nM),O-1918($GPR_{55/18}$; 1µM), (S)WAY (5-HT;300nM), SB366 ($TRPV_1$;500nM), CID (GPR_{55};1µM), and GW0742 (PPARδ;1µM).

In confluent cells, 70µM CBD, exerted significant cytotoxic effects with the same 60% reduction in cellular metabolism (P<0.0001, Figure ***17 B***). Interestingly, the combined application of the same receptor antagonists, amplified the already

significant cytotoxic effects of 70µM CBD by 20% (P<0.0001, ***Figure 17 B***). The application of 50µM CBDA provoked a significant 90% reduction in metabolism from the baseline, producing a noticeably more potent cytotoxic effect than previously observed (P<0.0001, **Figure 17 B** vs. P<0.001, ***Figure 16 B***). The addition of the combination of receptor antagonists had no detectable effect on the efficacy of CBDA. This same cytotoxic potency exhibited by CBD and CBDA was not observed under vehicle conditions and therefore it would be difficult to argue that the diluents, ethanol and acetonitrile (ACN), were responsible for the anti-cancer effects observed in these studies.

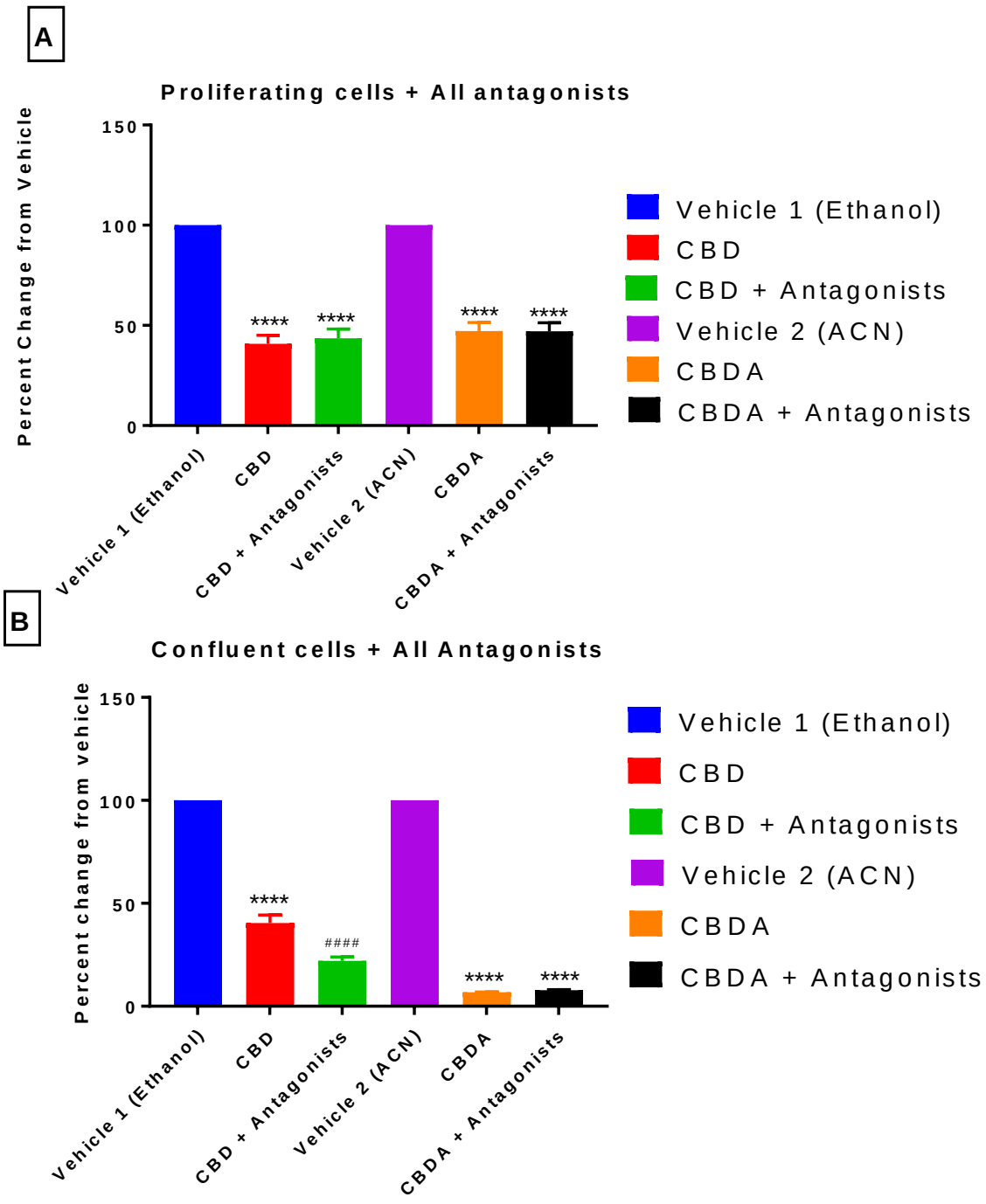


Figure 17. Effects of a combination of receptor antagonists AM251 (CB_1;100nM), AM630 (CB_2;100nM), GW6471 (PPARα;500nM), GW9662 (PPARγ;100nM),O-1918($GPR_{55/18}$; 1μM), (S)WAY (5-HT;300nM), SB366 ($TRPV_1$;500nM), CID (GBP55;1μM), GW0742 (PPARδ;1μM)) on the cytotoxic effects of 50μM CBDA and 70μMCBD when applied to proliferating **(A)** and confluent **(B)** cells for a 72 hour period (ACN = acetonitrile). Data expressed ± SEM. Statistical analysis conducted using one-way ANOVA, with Dunnett's(A) and Tukey's(B) *post hoc* testing; ****P<0.0001, ***P<0.001, **P<0.01,* P<0.05, (#) significant change from drug(**n=16**).

3.5 Total protein (BCA) and membrane integrity (LDH)

Following the promise of individual treatments of CBD and CBDA we aimed to investigate whether a combination treatment of the two compounds at previously identified therapeutic doses would further augment the anti-proliferative and cytotoxic effects observed in our previous studies. In a 12 well plate seeded with 1 x 10^5 cells/well, lysates collected from wells treated with 70µM CBD ($P<0.0001$) exhibited a 60% reduction in total protein after 72hr exposure compared to vehicle treated cells. Caco-2 cells treated with 50µM CBDA exerted a 40% reduction in total protein ($P<0.0001$) (***Figure 18 A***). Co-administration of CBD (70µM) and CBDA (50µM) to wells induced a 60% decrease in total protein ($P<0.0001$), comparable to the singular application of 70µM CBD.

Following analysis of cell lysates, media was analysed for the presence of LDH. As one can clearly see, the presence of 70µM CBD was significantly effective at inducing a cytotoxic leakage of LDH into the media of our samples ($P<0.0001$, ***Figure 18 B***). Similarly, the combined presence of CBD and CBDA (70µM and 50µM respectively) significantly increased the presence of extracellular LDH with similar potency to a lone treatment of 70µM CBD. The extracellular release of LDH in the presence of CBD and the combination treatment equates to the decrease in total protein observed in ***Figure 18 A***. This suggests CBD and CBDA treatments had cytotoxic effects on confluent CaCo-2 cells after single treatment and 72hr incubation. This same effect was not observed in the cells treated with when 50µM CBDA, a phenomena also observed in prior LDH analyses (***Figure 13***), with no

significant changes from the Vehicle conditions despite inducing a decrease in total protein (see figure, ***Figure 19 A***).

An enzyme linked immunoassay was performed, searching for the elevated presence of interleukin-6 in media samples in response to the cannabinoid and combination treatments (***Figure 19 C***). This IL-6 analysis yielded little significant data with only trace amounts detectable, falling below the bottom of the standard curve. This suggests a lack of IL-6 production from CaCo-2 cells under these conditions. The trace amount of IL-6 that were detectable did however show a minor increase in IL-6 in the presence of 70μM though this effect was not statistically significant.

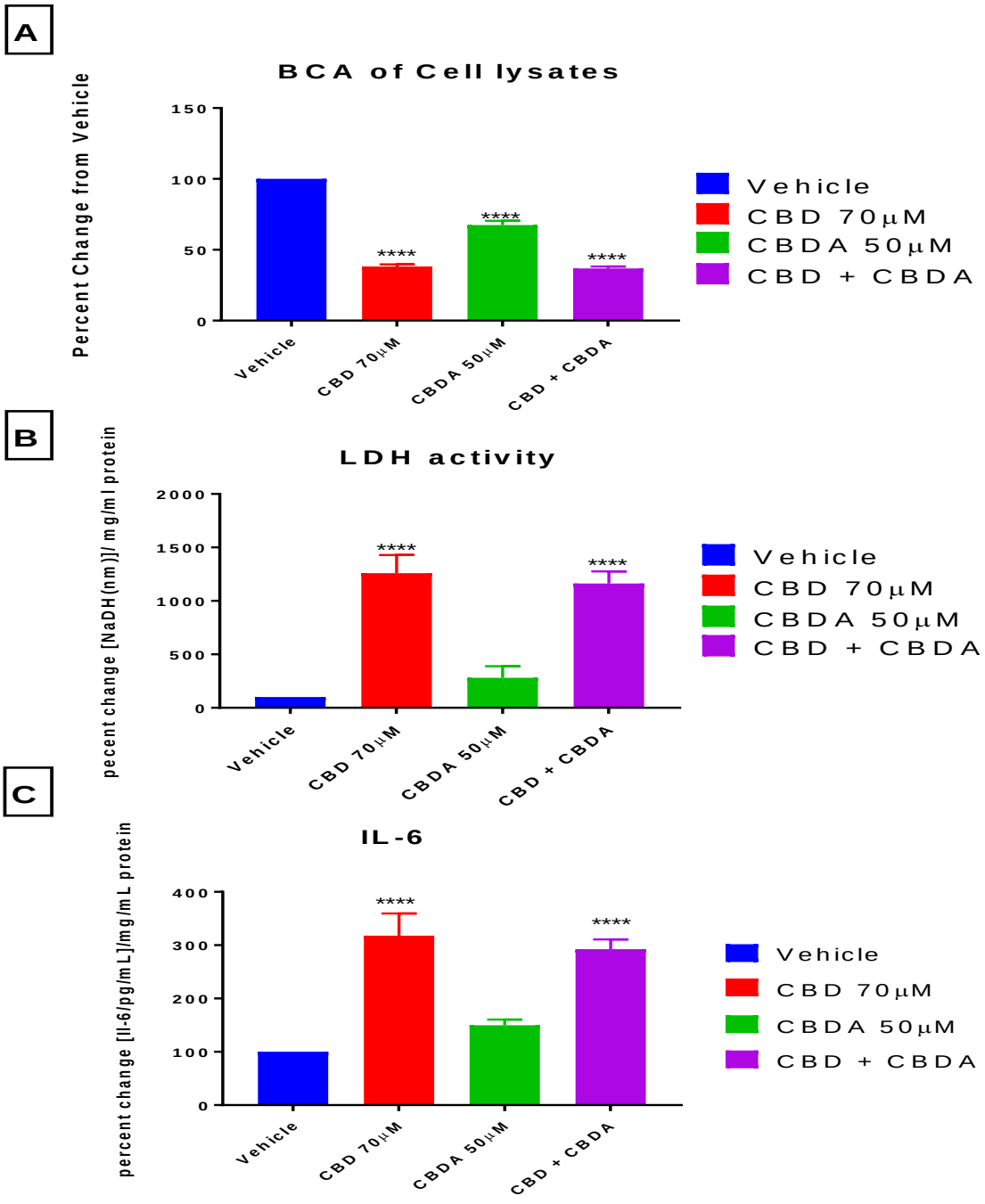


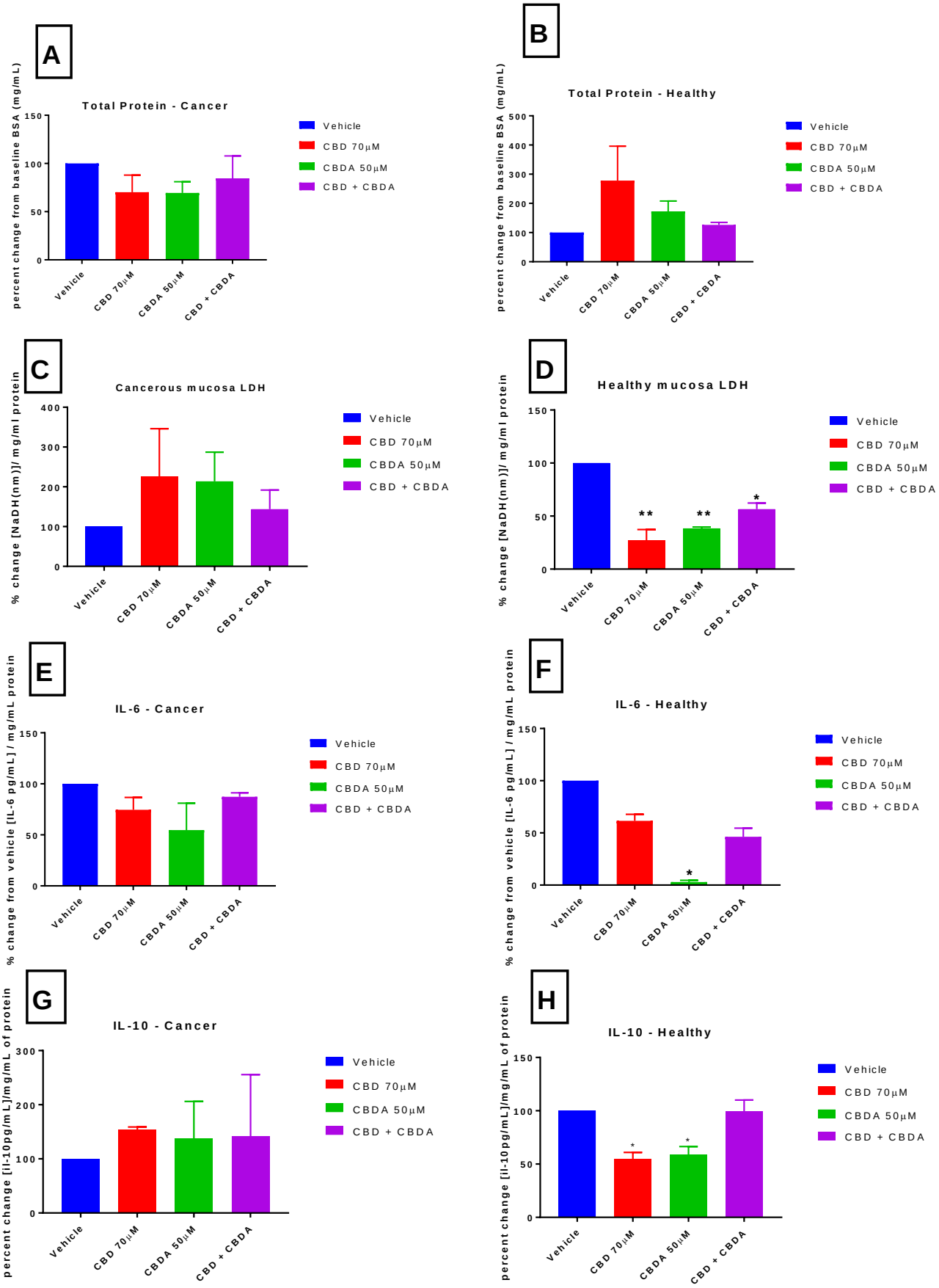
Figure 18. The effects of single and combination treatments of CBD and CBDA on cell lysates and media collected after 72 hours of treatment. Total protein quantified using BCA assay (**A**, **n=18**), cell damage analysed via LDH (**B**, **n=17)** and IL-6 production (**C**, **n=18**). One outlier removed from LDH data due to error in methodology. Data are given as means with error bars representing ± SEM. Statistical analysis of increasing concentrations against vehicle conducted using one-way ANOVA, with Tukey's *post hoc* testing. ****P<0.0001, ***P<0.001, **P<0.01, * P<0.05.(**n=9**).

3.6 CBD and CBDA effects in *ex vivo* Human colorectal cancer tissue

Exploration of the effects of CBD and CBDA provided multiple interesting observations. A variety of factors impeded the collection of human tissue samples and as a result, the data presented is derived from just two patients. The two donated patient samples were tested in triplicate, despite this, the data exhibits a great degree of variation. The total protein content tumour samples of after 72 hours of incubation with 70µM CBD, 50µM CBDA and a combination treatment did not exhibit any statistically significant change in the mg/mL of BSA (***Figure 19 A***). These same treatments of 70µM CBD, 50µM CBDA applied to the healthy tissue augment the BCA content of healthy tissue lysates by approximately 200% and 50% respectively (***Figure 19 B)***. This was observed to a far lesser degree in samples treated with the CBD and CBDA combination which increased total protein by 10%. The increases provoked by CBD and CBDA were not deemed statistically significant. As per the CaCo-2 trials, the effects of CBD and CBDA on extracellular LDH concentrations were once again assessed in the cancerous vs healthy tissue. The reduced BCA content caused by CBD and CBDA correlated to an increased concentration of extra-cellular LDH of 110% and 100% respectively (***Figure 19 C***). By contrast, the increase in BCA observed in healthy tissue, correlated to a significant reduction in extracellular LDH compared to the baseline. A significant reduction of 75% in the concentrations of extracellular LDH was observed in healthy tissue treated with 70µM CBD ($P<0.01$). A 60% reduction was observed in wells treated with 50µM CBDA ($P<0.01$), and a reduction of 40% in wells that received the combination treatment ($P<0.05$) (***Figure 19 D***). Interleukin 6 levels within these

tissue samples showed varying responses. Treated cancerous tissue exhibited a minimal capacity to reduce the pg/mL of IL-6 in each mg/mL of protein (***Figure 19 E)***. There was however a significant reduction in these levels in healthy tissue. Through media analysis we observed a significant >90% reduction in IL-6 production in the presence of 50µM CBDA ($P<0.05$). 70µM CBD provoked an insignificant 40% reduction in pg/mL of IL-6 in each mg/mL of protein, whereas the combination treatment reduced this by 55% (***Figure 19 D***). IL-10 expression followed a similar trend, with the cancerous tissue exhibited an almost two-fold increase in response to all treatments in one patient, this rise however was statistically insignificant due to a great degree of variation between the patient samples (***Figure 19 G)***. Healthy tissue treated with 70µM CBD ($P<0.05$), 50µM CBDA ($P<0.05$) demonstrated significant efficacy in reducing the expression of IL-10, the combination treatment did not have this same efficacy (***Figure 19 G vs H***). Though no significant results were obtained from the treatment of cancerous tissue, 70µM CBD ($P<0.05$) increased IL-17 production by 50% whereas, 50µM CBDA and the combination treatment provoked minimal responses (***Figure 19 I***). A significant 60% reduction in IL-17 was achieved in healthy tissue treated with 70µM CBD ($P<0.05$), and a reduction of 50% in tissue treated with 50µM CBDA ($P<0.05$), combination treatments had no significant response (***Figure 19 J***). The analysis of VEGF expression in the tumour tissue showed no significant results compared to the vehicle. CBDA slightly reduced the expression of VEGF by 50%. By contrast, CBD increased the VEGF expression by approximately 40% (***Figure 20 K***). In healthy tissue this VEGF expression negatively correlated to the cancerous tissue. CBD had

minimal anti-VEGF effects in the healthy tissue whereas healthy tissue treated with 50µM CBDA almost more doubled the VEGF expression in one patient (***Figure 20 L***).


A
Total Protein - Cancer
percent change from baseline BSA (mg/mL)
150
100
50
0
Vehicle
CBD 70µM
CBDA 50µM
CBD + CBDA
B
Total Protein - Healthy
percent change from baseline BSA (mg/mL)
500
400
300
200
100
0
Vehicle
CBD 70µM
CBDA 50µM
CBD + CBDA
C
Cancerous mucosa LDH
% change [NaDH(nm)]/ mg/ml protein
400
300
200
100
0
Vehicle
CBD 70µM
CBDA 50µM
CBD + CBDA
D
Healthy mucosa LDH
% change [NaDH(nm)]/ mg/ml protein
150
100
50
0
**
**
*
Vehicle
CBD 70µM
CBDA 50µM
CBD + CBDA
E
IL-6 - Cancer
% change from vehicle [IL-6 pg/mL] / mg/mL protein
150
100
50
0
Vehicle
CBD 70µM
CBDA 50µM
CBD + CBDA
F
IL-6 - Healthy
% change from vehicle [IL-6 pg/mL] / mg/mL protein
150
100
50
0
*
Vehicle
CBD 70µM
CBDA 50µM
CBD + CBDA
G
IL-10 - Cancer
percent change [il-10pg/mL]/mg/mL of protein
300
200
100
0
Vehicle
CBD 70µM
CBDA 50µM
CBD + CBDA
H
IL-10 - Healthy
percent change [il-10pg/mL]/mg/mL of protein
150
100
50
0
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*
Vehicle
CBD 70µM
CBDA 50µM
CBD + CBDA

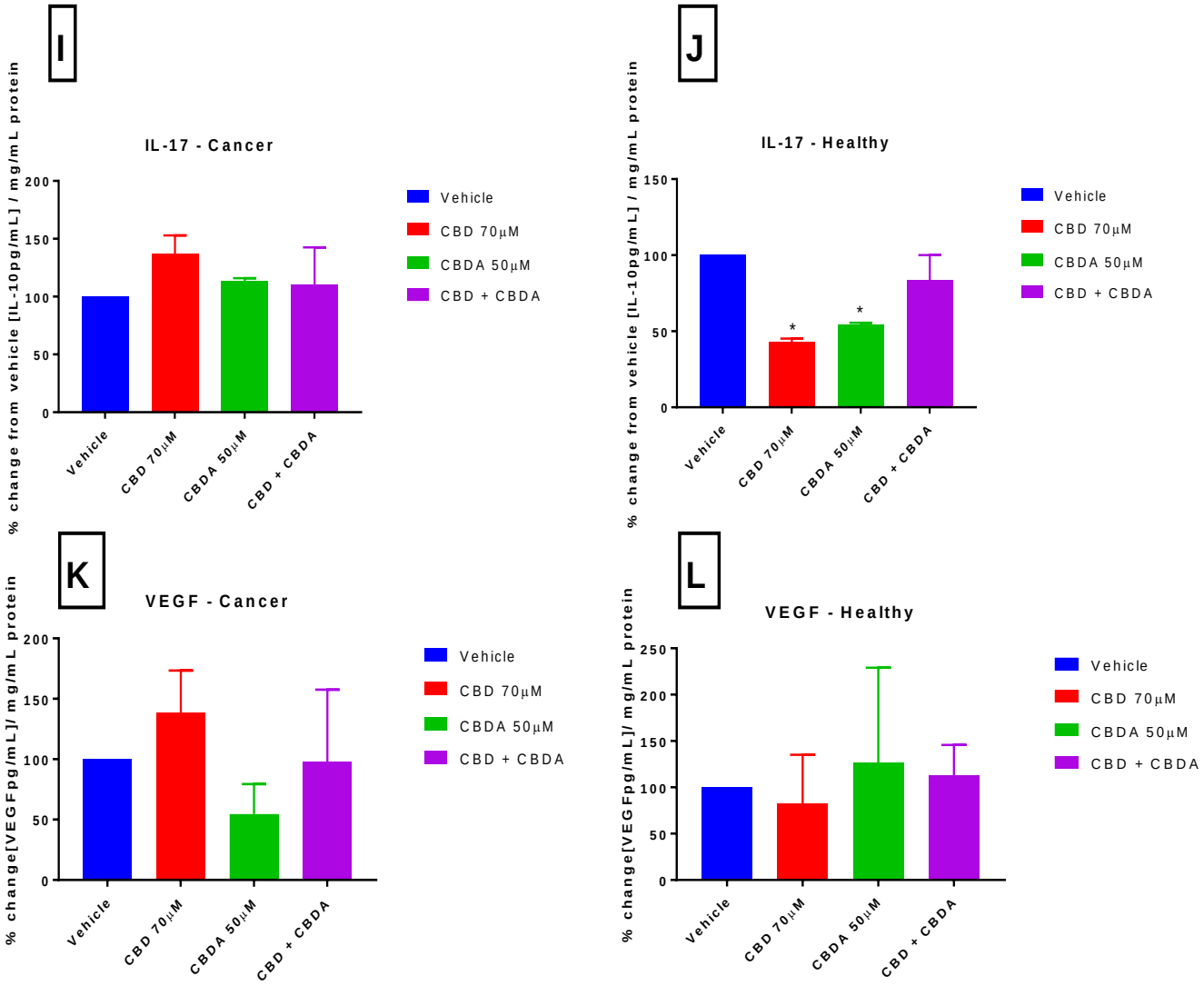
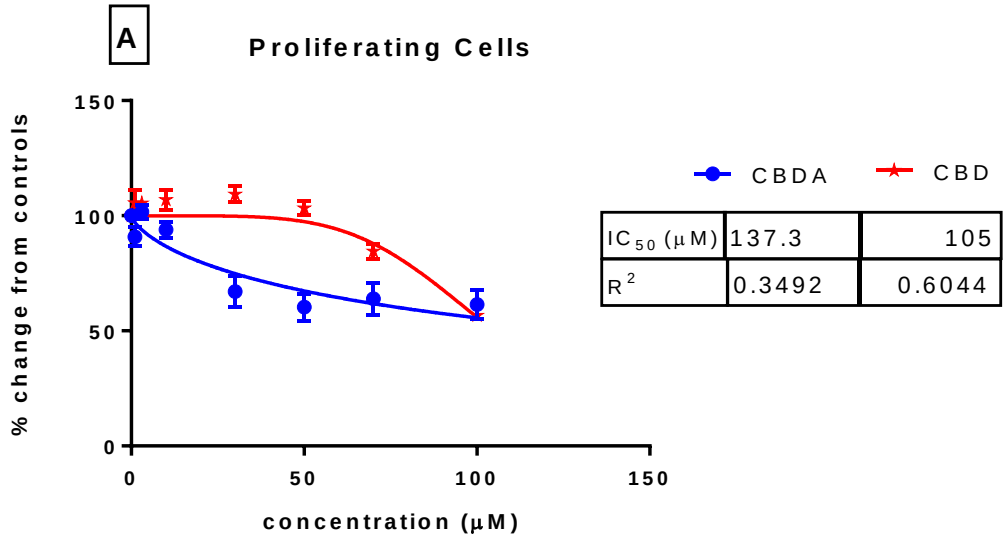


Figure 20. The effects of CBD and CBDA in ex vivo explants of cancerous tissue on total protein (**A**), LDH release (**C**), IL-6 (**E**), IL-10 (**G**), IL-17 (**I**), and VEGF (**K**) (left). Figures on the right hand side demonstrate the effects of CBD and CBDA in healthy tissue collected on total protein (**B**), LDH release (**D**), IL-6 (**F**), IL-10 (**H**), IL-17 (**J**), and VEGF (**L**). Data are given as means with error bars representing ± SEM. Statistical analysis of increasing concentrations against vehicle conducted using one-way ANOVA, with Tukey's *post hoc* testing. ****P<0.0001, ***P<0.001, **P<0.01, * P<0.05.(**n=2)**

3.7 CBD and CBDA Concentration Curves

Utilising the data procured over the course of our testing period, we developed a concentration response curve for CBD and CBDA in proliferating and Confluent CaCo-2 cells. Proliferating cells exhibited a higher degree of tolerance to the effects of CBD and CBDA requiring concentrations in excess of 100µM to achieve 50% inhibition, CBD was the more potent anti-proliferative (**Figure 21 A,B**). In confluent CaCo-2 cells CBD and CBDA were twice as toxic, with an IC_{50} values of 57.98µM and 51.45µM respectively.



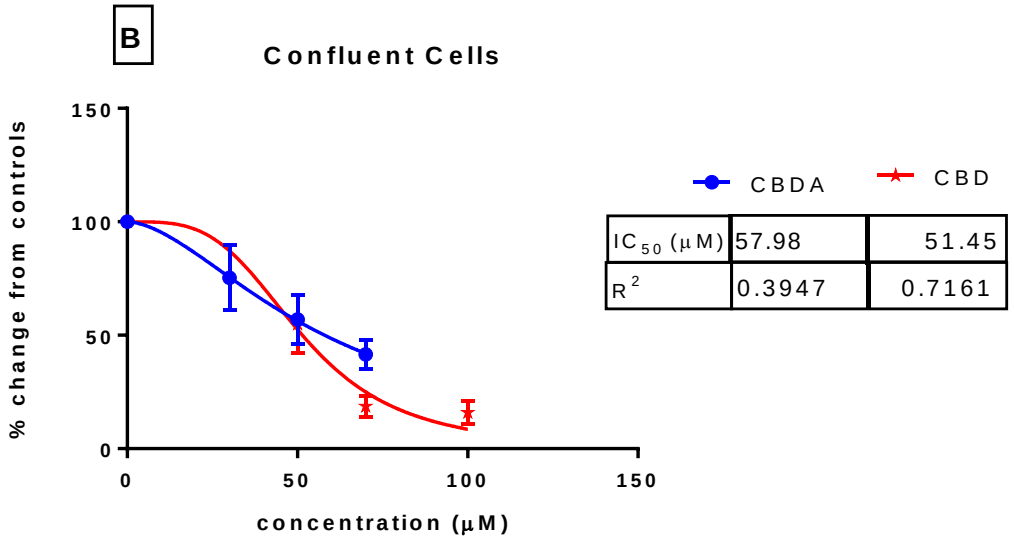


Figure 21. The concentration curves for CBD and CBDA in proliferating (**A**) and confluent (**B**) CaCo-2 cells following 72 hours treatment. IC50 values are presented above, with corresponding R-squared values.

4 Discussion

In light of the widespread cannabis use amongst cancer patients and the growing evidence of cannabinoid efficacy in cancer, we set out to demonstrate further potential anti-cancer effects of two cannabis derived compounds, CBD and CBDA, and study their effects in a human model of CRC (Waissengrin *et al.*, 2015; Karanges *et al.*, 2018; Martell *et al.*, 2018). In this paper we demonstrated the anti-proliferative and cytotoxic effects of CBD and CBDA in human colorectal cancer cell lines and explant tissue. The gap in existing literature suggested that various forms of cancer may benefit from cannabinoid treatment. As hypothesized, these experiments have demonstrated that CBD and CBDA have cytotoxic andante-proliferative effects in cancer cells, whilst reducing the inflammation associated with colorectal cancer. Using cannabinoid receptor antagonists, we identified several receptors which may contribute to the mechanistic actions of these drugs. As is expected for many novel therapeutics, for these molecules to be truly viable therapeutics, they must be seen to have significant benefit in disease progression and must importantly, improve or increase the efficacy of existing treatments.

Based on our assessment of existing literature (***Table 1***), we proposed CBD and CBDA as a potentially safer and more efficacious molecules which could be implemented alongside current chemotherapeutics and radiotherapeutics. Our previous work of has highlighted the anti-inflammatory ability of CBD in the gut *in*

vitro and *ex vivo* using inflammatory protocols (Couch *et al.*, 2017a). The strong correlation between cancer and inflammation, and the efficacy of CBD in alleviating cancer induced inflammation strongly supported the use of CBD in this context (Couch *et al.*, 2017a). Given the need for CRC therapeutics, we investigated the potential of CBD and CBDA in future CRC management strategies by investigating their anti-proliferative, anti-metastatic, cytotoxic and anti-inflammatory effects. In this section we discuss the promise and limitations of our findings in the context of current literature and discuss the use of these compounds in a wider therapeutic context.

4.1.1 Anti-Proliferative Efficacy

In this study we explored the anti-proliferative effects of CBD and CBDA at a variety of concentrations using a CaCo-2 cellular model of colon cancer to analyse the physiological responses of these cells to cannabinoid treatments. To date, literature has sparsely explored the efficacy of CBD and CBDA in CRC models and cell lines. Using CaCo-2 cells we reproduced the previously cited anti-proliferative effects for CBD (Aviello *et al.*, 2012a) . Further to this we observed novel anti-proliferative effects in cells treated with CBDA. The metabolic responses of CaCo-2 cells to CBD and CBDA were quantified by the resazurin salt assay. Using nanomolar and micromolar ranges for both test compounds, we determined the dose-response of these compounds in CaCo-2 cells. The CaCo-2 model explored prior to any explant tissue testing through which we aimed to identify, working concentrations for explant testing. In our CaCo-2 cell studies we were able to reproduce previously

observed anti-proliferative properties in cells treated with CBD and CBDA and report our own significant anti-proliferative effects over a 72hr treatment period. Anti-proliferative effects were observed in CaCo-2 cells treated with CBD at concentrations of 70-100µM. This reduction in proliferation was characterized by a significant reduction in the cellular metabolism of CaCo-2 cells as signalled by decreased resorufin production**.** CBDA demonstrated a higher degree of anti-proliferative potency than CBD, provoking significant anti-proliferative responses at concentrations as low as 30µM, ranging up to 100µM. Unexpectedly, CBDA exhibited more potent anti-proliferative effects in CaCo-2 cells than CBD, which is the first time this phenomenon has been reported. Under vehicle conditions, proliferation was un-inhibited strengthening our argument that it was CBD and CBDA that inhibited the proliferation of cells rather than the acetonitrile and ethanol that the drugs were dissolved in.

Later in these studies, antagonists were applied to these studies to explore the mechanisms of this anti-proliferative action. The anti-proliferative effects of CBD were partially inhibited in the presence of $GPR_{55/18}$ antagonist, 0-1918 and the GPR_{55} specific antagonist, CID-16020046 (***Figure 15 A***). We identified a number of receptor antagonists which were able to partially block the anti-proliferating effects of 50µM CBDA over the 72-hour testing period. The $TRPV_1$ antagonist SB366 and the GPR_{55} antagonist CID-16020046 significantly inhibited the anti-proliferative effects of 50µM CBDA at the 24, 48 and 72-hour time points (***Figure 15 B***). Two other antagonists, O-1918 (GPR55/18; 1µM), and (S)-WAY-100135 (5-HT; 300nM), inhibited the effects of CBDA at 24 and 48 hours respectively (***Figure 15 B***). Our observation of anti-proliferative effects in CBD treated CRC cells is corroborated by

previous findings in CaCo-2 and HCT116 colorectal cancer cell lines, these studies however, observed antiproliferative effects at concentrations of just 0.01µM up to 10µM (Aviello *et al.*, 2012b; Romano *et al.*, 2014b). Although we generated similar anti-proliferative findings to previous work in CRC cell lines, Romano *et al*, by contrast suggested that these effects may be occurring in a CB_1 sensitive manner. However, based on our data we propose CBD and CBDA may have a $GPR_{55/18}$ mediated function. Our identification of GPR_{55} is supported by work in two GPR_{55}-positive B-lymphoblastic cell lines which has shown activation of GPR55 has an anti-proliferative effect (Mangini *et al.*, 2017). The contradiction between our mechanistic findings and those of the Romano *et al* study may be due to variations in treatment ranges, Romano *et al*. observed anti-proliferative at much lower micro molar ranges of CBD (0.3 - 5µM), and in a different series of cell lines, DLD-1 and HCT116 colorectal carcinoma cells (Romano *et al.*, 2014a). In studies conducted by Aviello *et al,* in 2012, concentrations of 0.01 – 10µM CBD were applied to CaCo-2 and HCT116 cells resulting in a similar decrease in cellular proliferation, which again, was deemed to occur via the CB_1, $TRPV_1$, and PPARγ receptors, with no involvement of GPR_{55} or GPR_{18} (Aviello *et al.*, 2012). Though these studies observed similar anti-proliferative effects, the above studies by Aviello *et al* and Romano *et al*, both highlighted the implication of CB_1 which was not observed in our studies. GPR_{55} involvement has however been highlighted as an anti-proliferative mechanism in T98G, U251, and U87M glioblastoma cells (Deng *et al.*, 2017). This conflicting data again adds to the rationale for prioritising explant models for exploring mechanisms of action as cell lines can vary greatly depending on culturing methods and passaging of cells (Kaur and Dufour, 2012). One research study found

that GPR-55 may also play a tumour promoting role, where the levels of COX-2 and STAT3 were reduced in the tissue of GPR55 knockout mice (Hasenoehrl *et al.*, 2018). This same study highlighted an interaction between GPR_{55} and CB_1 and the dual capacity of these receptors to mediate elements of the endocannabinoid system. Further to this CB_1 has been shown to inhibit the signalling of GPR_{55} receptors, and by contrast, CB_1 receptor signalling is enhanced by the expression of GPR_{55} (Kargl *et al.*, 2012). Despite the initial conflict in data, it appears this link is more of an overlap in knowledge rather than a direct conflict in observations.

Our secondary anti-proliferative finding, that CBDA exerts a more efficacious anti-proliferative compound than CBD, is however, a novel observation. Though CBDA has previously been applied to MDA-MB-231 breast cancer cell lines at concentrations as high as 300uM, these studies only demonstrated the ability of CBDA to reduce inflammatory prostaglandin production by suppressing the cyclooxygenase enzymes COX-1 and COX-2 (Ruhaak *et al.*, 2011). Previous CBDA studies by Takeda *et al,* were unable to elucidate any mechanisms of action in the anti-proliferative action of CBDA in MDA-MB-231 breast cancer cells (Takeda *et al.*, 2012, 2014, 2017). Given this lack of mechanistic exploration, our proposition is that the $TRPV_1$, GPR_{55}, GPR_{18} and the 5-HT_{1A} receptors may be implicated in the mediation of the anti-proliferative action of CBDA. Our studies are the first to utilise receptor antagonists in this manner and suggest several novel mechanisms for the anti-proliferative effects of CBDA. These results may be significant in future studies looking to explore the use of these compounds alongside current chemotherapeutic and radiotherapy strategies. These studies also provide a structure and point of reference for further exploration of the effects of CBDA and CBD in other forms of

cancer. Our proliferation studies have highlighted that CBDA may indeed be a more efficacious drug in the inhibition of CRC growth, as well as providing further rationale for the wider exploration of the effects of compounds such as this which as yet have been underutilised in cancer research.

4.1.2 Cytotoxicity

Our investigation into the ability of CBD and CBDA to cause cell death in CRC tumours initially utilised a confluent Caco-2 cell model to simulate a tumour mass of cells. Physiological responses were again quantified using the resazurin assay technique. The resazurin assay was used alongside a lactate dehydrogenase (LDH) assay allowing for assessment of the plasma membrane integrity in response to our drug treatments (Chan *et al.*, 2013). When treated with CBD at concentrations of 70 and 100µM, the metabolic activity of confluent cells was significantly inhibited after 24 hours and maintained until the termination of the study period. This reduction in metabolic activity correlated to a loss of membrane integrity causing a subsequent increase in extracellular LDH. Conversely to the proliferation studies, these toxicity studies demonstrated that CBD was the more efficacious of the two compounds in terms of reducing cell viability and reducing cancel membrane integrity. CBDA demonstrated weak cytotoxic effects compared to CBD and lacked the same toxicity in confluent cells. This is interesting given other studies exploring the effects of *Cannabis sativa* ethanol extracts containing CBDA have shown the drugs ability to reduce the viability of HCT 116 colon cancer cells and have cytotoxic effects on human polyp biopsies (Nallathambi *et al.*, 2018). Interestingly however, the initial treatment of cells with CBD at the 0hr time point caused an increase in

both the metabolic activity of the CaCo-2 cells, provoking a marginal increase in LDH. These same phenomena occurred at the 0hr time point in confluent cells treated with CBDA which increased metabolism by 100% and resulted in a 50% decrease in extracellular LDH. Cells were serum starved prior to seeding, incubated for 3 days, then treated with CBD and CBDA. During this 3-day incubation period it is likely that the glucose levels in the cellular media may have depleted and therefore the initial increase in metabolism that we observed in these studies may have been the result of glucose intake from media containing our drug treatments. The Crabtree effect describes the increased utility of carbohydrates by cancer cells resulting in a decrease in oxygen consumption by cancer cells (Crabtree, 1929). This transformation in metabolism is thought to be a characteristic required for rapidly growing cells, an effect often observed in spermatozoa and stem cells (Fadaka *et al.*, 2017). In light of this, and the absence of this starvation period immediately prior to treatment, cells may have become hyper sensitive to the addition of fresh media potentially explaining the delay to the effects of the drug treatments (Pirkmajer and Chibalin, 2011). Another explanation for the initial increase in metabolic activity upon drug treatments may be that an initial increase in metabolism is in fact needed to synthesize degradation proteins and prepare the cells prior to cell apoptosis.

Following these studies, we aimed to explore the complementary anti proliferative effects of CBD and CBDA. Another series of 72-hour studies were conducted, exploring the effects of combination treatments of CBD and CBDA. With CBD exhibiting high cytotoxic efficacy and CBDA demonstrating high anti-proliferative potency, we explored whether a combination of both drugs, added to

a series of 12 well plates containing CaCo-2 cells, would augment the potency of the observed anti-cancer effects. The efficacy of this combination treatment was compared to lone treatments of CBD and CBDA. The combination treatments demonstrated much the same effect as the lone CBD treatments. Combining CBD and CBDA did not augment the effects compared to CBD or CBDA alone. The reduction in the quantity of BCA in cancer samples treated with CBD implies that a significant reduction in cancer cell distribution was achieved with CBD treatments. This, coupled with a significant increase in the extracellular levels of LDH, strengthens the argument that CBD was exerting cytotoxicity. The decrease in total BCA and increase in LDH, correlated to an increased concentration of IL-6 in the media collected from. The increase in IL-6 was unexpected and contradicted our initial hypothesis but offered a reference as to how these drugs may function in our explant tissue. This increase in IL-6 expression suggests CBD in this model may be mediating inflammation via the upregulation of the STAT3 and NF-kB transcription factors (discussed in **4.2**). Although we were not able to quantify these elements in our experiments, elsewhere, combination treatments of cannabinoids have previously been shown to be more efficacious than the application of lone cannabinoids, possibly due to other phytocannabinoids interacting synergistically for cytotoxicity in colon cancer cells (Nallathambi *et al.*, 2018).

In our antagonist studies, we aimed to elucidate the mechanisms underlying the observed anti-proliferative and cytotoxic effects. However, we were unable to isolate the implication of any of the individual cannabinoid receptors. This suggests several possibilities. One possibility is that the antagonist concentrations used in this study may have been insufficient to fully inhibit receptor activity. The second

possibility is that, CBD and CBDA may not bind specifically to any one of the receptors explored, and therefore inhibition could not be achieved. The third possibility is that the antagonists may overlap somewhat and show receptor promiscuity.

Most interestingly however, the addition of CB_2, GPR55/18 and 5-HT_{1A} antagonists augmented the cytotoxic effects of CBD. Though this result was the opposite of the desired result, it suggests that these receptors may still have some degree of implication in the modulation of cell apoptosis. These same receptors showed some degree of modulation in the proliferation studies and so we expected some degree of overlap. Sister cells treated with CBDA did not exhibit the same degree of toxicity as CBD treated cells. CBDA treatments only exhibited toxicity at a concentration of 70 µM at the 72hr time point. Similarly, the only significant increase in LDH production occurred at 70 µM CBDA but instead at the 48hr time point. In follow-up antagonist studies CBDA's mild toxicity was inhibited by the antagonism of the GPR-$_{55}$, 5-HT_{1A} and PPARδ. Again, we highlight the involvement of GPR_{55} and 5-HT_{1A} in the cytotoxicity of these compounds. These findings suggest CBDA has some degree of involvement amongst those receptors, however, as we did not perform a lone study solely exploring the effects of the antagonists, we cannot rule out the antagonists having pro-cancer effects.

Another novel observation made in this paper was that both CBD and CBDA have exhibited cytotoxicity that seems to be modulated, at least in part, by GPR_{55} and 5-HT_{1A}. Conclusions drawn by Deng *et al* also suggest the involvement of GPR_{55} in glioblastoma cell lines in which CBD reduced cell viability as well as a decreasing

proliferation. These glioblastoma studies also linked CB_1 and $TRPV_1$ to the anti-proliferative and cytotoxic action of CBD (Deng *et al.*, 2017). We repeatedly linked the GPR_{55} receptor to the anti-proliferative and cytotoxic effects observed in our study. One paper has demonstrated however highlighted that the CB_1 antagonist AM251 can activate the GPR_{55} receptor and so we must be careful when claiming that GPR*55* is directly implicated in the mechanistic action of CBD and CBDA (Ryberg *et al.*, 2009). The unwanted activation of GPR_{55} by AM251 can however be avoided in future work by using another CB_1 antagonist, AM281 (Ryberg *et al.*, 2009). Though this paper may stifle the conclusion that the anti-cancer effects we observed are GPR_{55} mediated, other studies have shown atypical cannabinoids, such as O-1602, and abnormal cannabidiol, have the capacity to activate GPR_{55} in HEK293T embryonic kidney cells (Johns *et al.*, 2009). Despite the minor degree of uncertainty added to our results by Ryberg *et al*., this research was conducted in a very different model to the above literature, which was not cancer focussed. Though our data highlighted the implication of GPR_{55} in the mediation of CBDA's effects. This finding is somewhat contradicted by a 2017 study conducted by Takeda *et al*. on MDA-MB-231 breast cancer cells in which it was concluded that the decrease in the cancerous C-Fos expression was, specifically, not mediated by GPR_{55}. This is however the only other study from our literature review that was conducted in a cancer cell line that may have grounds to dispute our conclusions. GPR_{55} has long been proposed as a cannabinoid receptor and so our conclusion that GPR_{55} mediates the effects of CBD and CBDA in some part is not beyond the realms of possibility (Lauckner *et al.*, 2008; Ryberg *et al.*, 2009). In addition to GPR_{55}, CBD and the $5\text{-}HT_{1A}$ receptor have previously been shown to interact with one another,

where the agonistic binding of CBD has exhibited various downstream effects. A review performed by Russo *et al*, examined the agonistic properties of CBD at 5-HT_{1A} serotonin receptor, concluding that, CBD indeed has a modest affinity for human 5-HT_{1A} receptors and that again further work was needed to clarify further effects at other serotonin receptor families. Of the cancer related literature reviewed at the start of this paper, our study is the first in this field to highlight this potential mediating role of 5-HT_{1A} receptor binding in CRC cells (Resstel *et al.*, 2009; Espejo-Porras *et al.*, 2013). Similarly, CBDA has also been linked to 5-HT_{1A} receptor activation, though not in a cancer model, this was observed in a study exploring the ability of CBDA to reduce vomiting in rats (Bolognini *et al.*, 2013). Our findings show that CBDA is more potent at 5-HT_{1A} receptors than CBD.

Further to the GPR_{55} and 5-HT_{1A} receptors and there is strong support for the involvement of PPARδ in CBDA signalling. The peroxisome proliferator-activated receptor (PPAR) family are a group of nuclear hormone receptors with three isoforms, all of which were explored in our antagonist studies (Alexander *et al.*, 2015). Nadal *et al* show that the acid forms of Δ9 -THC and CBD (Δ9 -THCA and CBDA) have a higher affinity for PPARγ than Δ9 -THC and CBD. Thus, this may explain why PPARδ involvment was only observed in CBDA treated cells (Nadal *et al.*, 2017). PPAR's, such as PPARγ have been linked to the anti-proliferative effects of CBD in CaCo-2 and HCT116 cells (Aviello *et al.*, 2012). Several studies support our conclusions that GPR-55, 5-HT_{1A} and PPARδ are involved in the potentiation of the CBDA and CBD's cytotoxic effects. However there remains a need for definitive exploration of the anti-cancer mechanisms of these phytocannabinoids in order to broaden their use.

Following the initial antagonist studies and failure to fully inhibit the anti-proliferative and cytotoxic effects of CBD and CBDA, we used a combination of all the antagonists. This was done in order to see if a combination could produce any further inhibition of the drug efficacy. Based on our ability to partially inhibit the effects of CBD and CBDA with single antagonists, we aimed to generate further signal suppression by using a combination of antagonists. This combination of antagonists had no significant effect on the ability of CBD and CBDA to inhibit proliferation. In confluent cells however, the combination of antagonists augmented the cytotoxic effects of CBD suggesting antagonism of one or more of these receptors may be of potential benefit in increasing the efficacy of CBD treatments. Of note is the observation that CBDA exhibited higher cytotoxic efficacy in this study than in initial cytotoxicity studies which may have been caused by inconsistencies in the dilutions of the drug or differences between cell passages. Considering this, it is apparent that significantly more clarity is needed regarding the mechanism underlying the effects of CBD and CBDA. Our studies go some way towards contributing to this knowledge gap, but as previously mentioned there is a still a great deal of exploration required to fully piece together the complex interaction of cannabinoids within the ECS.

4.2 *Ex vivo* CBD and CBDA

Following the acquirement of ethical approval, we commenced studies into the wider effects of CBD and CBDA on the expression of interleukins and growth factors. As outlined in section 2.4, we utilised explant tissue from patients undergoing colonic resections. Unfortunately, these studies were limited in their scope due to multiple unfortunate delays which impeded the collection of vital

data. The nature of this research meant that we experienced a variety of ethical constraints in the collection of serum and tissue samples from volunteers. With this study being performed by a master student, the study was limited by researcher training and knowledge. Difficulties were also experienced in sourcing tumours of a suitable stage, resulting in the collection of just two tissue samples (n=2). Due to the low sample number, there is significant variation in the data and so observations made in these explant studies are of low generalizability.

Despite the pitfalls of clinical research, we knew the use of more physiologically representative CRC models was being sought by international research teams, and so by using this model we aimed to contribute with more physiologically relevant data, modelled by treating human biopsies of CRC tumours with novel therapeutics (Novo *et al*., 2017). Our previous work utilised this same explant model to assess the anti-inflammatory effects of CBD on the acutely inflamed colon (Couc*h et al*., 2017). Furthermore this model had been previously used in literature to assess the chemotherapeutic, Selumetinib, which demonstrated efficacy in 22% of patient samples at concentrations ranging from 0-3µM (Novo *et al*., 2017). We aimed to compare the efficacy of cannabinoids to current chemotherapeutics using models that were already recognised as physiologically relevant. One benefit of explant models is that *ex vivo* samples contain lymphoid aggregates and innate immune cells which are absent in CaCo-2 cells (Jarry *et al.*, 2015). Despite the differences between models, the aim of our initial work in CaCo-2 cells was used to develop an outline of what to expect in the explant studies. Having already tested the same treatments in CaCo-2 cells, we hoped to be able to draw some comparison between the results of the CaCo-2 cells

to *ex vivo* CRC tissue. Protein analysis from the initial CaCo-2 cell combinations treatments (see section 3.5) exhibited a string of similarities to the data collected from the cancerous tissue explants. Analysis of the BSA content from cancerous tissue highlighted a similar reduction in the total protein content of these samples, this effect was not as pronounced as in CaCo-2 cell studies and did not show statistical significance. CBD treatments in healthy tissue samples resulted in an almost three-fold increase in total protein whilst CBDA treatments resulted in just under double the total protein content. The increase in total protein observed in healthy tissue explants, though visually impactful, lacked statistical significance. This may again have been a result of the low sample number and lack of data points. These alterations in total protein content correlated to some interesting findings in the levels of extra-cellular LDH. The minor reduction in total protein of cancerous tissue correlated to an increase in the levels of LDH released extracellular suggesting a degree of cytotoxicity in response to CBD and CBDA treatments. The opposite was observed in healthy tissue in which all three treatments, CBD, CBDA and the combination of CBD and CBDA, improved the integrity of cellular membranes. This equated to a reduction in the expression of extracellular LDH, whilst simultaneously increasing total protein.

In addition to its existence in serum, IL-6 expression is typically raised in CRC tumours compared to healthy tissue, based on this we expected to see a pronounced decrease in IL-6 concentrations in response to CBD and CBDA (Zeng *et al.*, 2017). However, our drug treatments provoked a varying response *in vitro* compared to *ex vivo.* The analysis of IL-6 in our Caco-2 study suggested that the addition of CBD and CBDA may augment the production of IL-6 once applied to

explant tissue. This, however, was not the case. IL-6 expression in cancerous tissue was marginally reduced in the presence of CBD and CBDA treatments. A similar effect was provoked in the healthy tissue, 50µM CBDA showed significant efficacy in reducing the expression of IL-6 to almost undetectable quantities. 70µM CBD and the combination treatment demonstrated weak anti-IL-6 potential but failed to provoke a significant effect.

Other key interleukins in explant tissue demonstrated a similar trend. Interleukin-10 is an immune modulator protein which has both pro-tumour and antitumor effects. As discussed in 1.5.2, IL-10 is considered a marker of poor prognosis in CRC, but interestingly lower IL-10 levels are associated with a higher risk of the disease (Abtahi *et al.*, 2017). This dual association with CRC meant that Interleukin 10 was of particularly interesting in this study. Interleukin 10 concentrations in cancerous tissue rose slightly in response to all treatment cases but this increase lacked statistical significance. It is unclear whether this was a beneficial or an adverse response, however, healthy explant tissue demonstrated and the opposite response. 70µM CBD and 50µM CBDA significantly inhibited the expression if IL-10 whereas our combination treatment demonstrated no efficacy. This same trend was observed in the expression of interleukin 17. Again, cancerous tissue exhibited no significant responses to our cannabinoid treatments. In healthy tissue, however, we again observed that 70µM CBD and 50µM CBDA significantly inhibited the expression if IL-17 whereas our combinations treatment did not. IL-17 though still debated, is typically considered to be a promoter of CRC, and so we aimed to inhibit the expression of this cytokine (Wu *et al.*, 2013). Though this was successful in healthy tissue, the significance of increased IL-17 in cancerous tissue

following CBD and CBDA treatments is unclear. Both interleukins were clearly affected by the application of CBD and CBDA, however, without a broader view of the impact of these findings on other downstream cytokines it is difficult to speculate as to whether the effects we observed would be beneficial in patients.

VEGF is known to contribute to the angiogenesis of cancer cells leading to tumour maturation and ultimately metastasis (Carmeliet, 2005). The importance of VEGF in CRC cannot be understated as, approximately 50% of CRC tumours exhibit VEGF expression which is otherwise absent in healthy tissue (Choi *et al.*, 2008). A significant amount of variation was observed between VEGF concentrations in our patients. Unfortunately, as we were unable to collect data from the patient notes of each volunteer, we are unable to draw comparison between the cancer staging of our donors. Based on existing literature, higher VEGF levels are typically expected in hypoxic environments, such as those found within tumours and so we expected that the cancerous tissue would contain higher basal VEGF concentrations. In these VEGF studies, the responses detected in healthy and cancerous tissues varied greatly. Again, these treatments did not provoke statistically significant effects. However, 70µM CBD stimulated a discrete increase in VEGF whereas 50µM CBDA caused a slight reduction, with the combination treatment mimicking the effects of the CBD alone. Healthy samples did not respond to CBD treatments or combination treatments, 50µM CBDA in this case increased VEGF in one of our healthy participants. Had the sample size have been larger and had patient history data have been available, we would likely be able to draw more substantial conclusions from this study. These findings, although lacking in numeracy, provided valuable insight into the interactions of CBD and CBDA in human physiology. Though these

findings are based on a test population of just two, studies conducted in *ex vivo* models offer a more physiologically relevant representation of how CRC tumours may respond to cannabinoid treatment *in vivo*. Previous work of ours supports several of our *ex vivo* discoveries regarding the inhibition of interleukin expression. A prior study of ours conducted in a model of the acutely inflamed colon, utilised an inflammatory protocol to artificially induce an inflammatory state. Our previous work demonstrated the ability of CBD to inhibit the interleukin production caused by an artificial inflammatory protocol. Our previous findings demonstrated the ability of CBD to reduce the expression of IL-6 and IL-17, which we recreated in healthy explant tissue. However, we were unable to exhibit this effect in cancerous tissue. Although we were unable to reproduce the anti-IL-6 effects of CBD in our cancerous samples, we demonstrated that CBD could reduce the concentration of total protein in cancerous samples. Furthermore, we showed that this reduction in total protein correlated to a decrease in the membrane integrity of treated cancer cells. This suggests the application of CBD and CBDA may be cytotoxic in cancerous tissue. The opposite was observed in healthy tissue, with an increase in total protein and a decrease in the extra cellular LDH. This finding in conjunction with the cytotoxicity observed in cancerous tissue, suggests CBD and CBDA may have a non-toxic effect in healthy tissue and may aid cell health. Peripheral tissue from tumours is known to exhibit inflamed properties and so it appears that CBD and CBDA were able to attenuate this augmented inflammation (Fisher *et al*., 2014).

These *ex vivo* studies highlighted that CBD and CBDA are able to target cancerous tissue whilst sparing healthy cells. This observation is supported by our media analysis for Interleukins. The ability of CBD and CBDA to significantly inhibit

the production of IL-6, IL-10 and IL-17 in healthy tissue but not cancerous tissue is an unexpected finding. Literature cited the overexpression of various interleukins in tumour samples which our ELISA's confirmed the presence of, but we were unable to affect the levels of these cytokines in cancerous tissue. It is possible that the loss of membrane integrity may have reduced receptor availability and therefore weakening the therapeutic response to CBD and CBDA (Wang *et al*., 2014). This inability of CBD and CBDA to interrupt interleukin levels in the cancerous tissue may however be of benefit. Previous work by Amicarella et al, has highlighted a dual role for IL-17 and the cells that produce it. Despite the previously reported pro-inflammatory action of IL-17, it additionally aids in the recruitment of beneficial immune cells such as neutrophils and cytotoxic CD8+ T cells (Amicarella *et al.*, 2017). Given this data, it may be therapeutically beneficial that CBD and CBDA were unable to inhibit interleukin production and instead heightened the expression of IL-17 other interleukins may serve as a beneficial immune response to CRC cells.

Despite the inability of CBD and CBDA to exert anti-interleukin activity in cancerous tissue, these compounds still elicited cytotoxic properties in the cancerous samples whilst sparing healthy (proximal) tissue. We argue that anti-interleukin and non-toxic effects of CBD and CBDA on proximal tissue and the cytotoxic effects in cancerous tissue, justify further exploration of these compounds as cancer therapeutics. Although we did not observe anti-interleukin activity in the cancerous tissue, studies support the theory that acute inflammation exerts an immunoprotective response, and the absence of anti-interleukin activity, in this case, may promote tumour eradication (Setrerrahmane and Xu, 2017). It is still unknown as to how interleukins and their receptors promote tumorigenesis (Wang

et al, 2014). Interleukin 17 is broadly distributed in the human colonic epithelium, is a promoter of angiogenesis and is associated with a poorer disease prognosis (Liu *et al.*, 2011; Harvey *et al.*, 2014). In our studies we demonstrated that CBD and CBDA could be used to reduce the concentrations of these IL-6, IL-10 and IL-17 when applied to what we referred to for the purposes of this study as healthy tissue. If in future studies we can demonstrate that CBD and CBDA treatments inhibited subsequent STAT3 and NF-kB transcription factor activation by the inhibition of these interleukins, then we may have a particularly promising argument for the use of these compounds as anti-cancer drugs (Waldner *et al*., 2012). As previously mentioned, the ability to reduce the levels of interleukins 6 and 17 offers great benefit in reducing the viability of cancer cells, we were able to demonstrate this in explant tissue. Multiple works have highlighted the therapeutic potential of targeting this interleukins, we were able to achieve this with micro molar doses of CBD, as well as showing that this can also be achieved with CBDA (Kumari *et al.*, 2016; Taniguchi and Karin, 2018). Although, our *ex vivo* findings are supported by our previous experimental data showing the efficacy of CBD as an anti-interleukin compound and highlight the efficacy of these compounds as CRC therapeutics to be utilised alone or as adjuvants to traditional chemotherapy (Couch *et al.*, 2017a). It must be remembered that this analysis has been drawn from a test group of just two, and so conclusions drawn from this work should not be over stated. As previously mentioned, this data is limited, but offers exciting insight in how human tissue acutely reacts to CBD and CBDA. Though these treatments hold great promise as a method of cancer therapy, our findings are a long way from being implemented as a conventional anti-cancer therapy, still requiring in depth

investigation before a refined therapeutic can be developed (Grivennikov and Karin, 2011).

4.3 Study Limitations

The CaCo-2 model is a well-established model for the analysis of the intestinal barrier, which happens to be a cell lineage derived from human colon carcinoma (Sambuy *et al.*, 2005). Though this model is well established, *in vitro* cell lines do have their drawbacks. These human derived cell lines are known to behave differently to primary *ex vivo* cells, which we observed when comparing the capacity of IL-6 production between CaCo-2 cells and human tissue (Kaur and Dufour, 2012). The culturing of cell lines is a time sensitive procedure which can be heavily influenced by the percentage confluence at which cells are sub cultured. For our *in vitro* studies, we sub cultured CaCo-2 cells at 80% confluency as advised by the supplier. However, studies observing the effects of cell seeding density on the structural and functional properties of CaCo-2 cells found that, cells cultured at 50% confluency retained a higher proliferation potential and developed a more homogenous and polarized cell monolayer (Natoli *et al.*, 2011). We also noted that the use of physiological levels of O_2 had been found to sensitise the CaCo-2 model to CBD. Unfortunately, we were constrained by the capacity of our culturing facilities and due to time constraints elected to base our procedure on our previous studies. As a result of this, for our studies we elected to utilise the manufacturers recommended sub culturing guidance, with the knowledge that these conditions would yield consistent results. Although every precaution was taken to maintain

consistent sub culturing conditions, variations may also have occurred as a result of inconsistencies in the media formulations, which can alter the perfusion of cell cultures and subsequent morphology of our cells. Despite these drawbacks, the CaCo-2 cell model utilised in this research paper was performed with much of this knowledge in mind. The data derived from these studies showed a high degree of reproducibility and exhibited the same trends across various cell passages using numerous repeated studies. The concentrations explored in this study, ranged from 1 to 100µM. A 100µM concentration (11.31 µg/mL) would be attainable in human serum, such concentrations would be comparable to the therapeutic dose for paracetamol which is 10-25 µg/mL (Schulz *et al*., 2003). Though this study did not directly explore modes of administration, the therapeutic concentrations achieved in these experiments could be achieved in the plasma utilising various means of administration, whether that be orally or intravenously.

Our use of the human explant model provided a more physiologically representative method of exploring the potential of CBD and CBDA compared to the CaCo-2 model. Though the findings from our *ex vivo* model are extremely valuable, the recruitment of just two patients over the testing period meant that our data offers little in the way of generalisability. However, what little data was collected, provided a unique opportunity for comparing how cancerous tissue and healthy respond to cannabinoid treatments. Although this was produced in a sample size of just two, we were successfully able to alter the levels of our target cytokines and proteins from baseline conditions with CBD and CBDA treatments. Despite these responses offering little in the way on conclusions, it does support the use of *ex vivo* tissue and its ability to retain physiological functions.

Another short fall of this study was that we did not explore the independent effects of our receptor antagonists in the absence of CBD and CBDA. One issue we experienced as a result of this is that we were unable to determine whether the inhibitory and excitatory effects we observed were a direct result of the drug treatments themselves or whether the receptor antagonists had induced independent anti-cancer effects. We did not derive our own working concentrations using dose response curves for each antagonist due to time constraints and relied upon previously reported working concentrations (Couch *et al.*, 2017b). Without this data, the unknown influence of antagonists and their potential toxicity, or anti-proliferative effects cannot be ruled out, and so this does limit the mechanistic conclusion that can be made. Independent antagonist's data may for greater insight into how endocannabinoid receptors may be targeted therapeutically.

4.4 Future Directions

Future work in this field has a wealth of therapeutic avenues to explore. Studies investigating the wider relationship between cannabinoids and the endocannabinoid signalling pathways, including variations in endocannabinoid tone would be of significant benefit to exploration in this field. In future work, high through put analysis using Multiplex testing should be prioritised to maximise the analytic capabilities for observing the physiological responses of cytokines and growth factors to cannabinoid treatments. Multiplex analysis would increase the speed with which samples could be analysed and reduce many of the labour intensive aspects of the analytical process (Elshal *et al.*, 2006). Another avenue of exploration would be analysing the responses of ex-plant tissue to multiple or

repeat cannabinoid treatments using high-throughput multiplex analysis of established pro-inflammatory cytokines, growth factors and caspases. Exploration of these effects could further our understanding of how the efficacy of cannabinoid treatments could be increased with repeated doses or in combination with current chemotherapeutics. We made several early attempts to investigate whether a breast cancer chemotherapeutic would show efficacy in our CaCo-2 cells. The aim of this was to see how cannabinoids may increase the efficacy of existing chemotherapeutics and exhibit potential as an adjuvant therapy. Unfortunately, however, we were unable to provoke any chemotherapeutic responses in our cells using this compound. Future work may wish to explore this theory using chemotherapeutics specific to CRC cancers such as Selumetinib, which is currently being used alongside Cyclosporin A in order to increase the efficacy of the treatment (Krishnamurthy *et al.*, 2018). Based on our investigation of CBD and CBDA, the combination of these drugs with Selumetinib may demonstrate their use as an adjuvant therapy to combat the inflammatory effects of chemotherapy (Braun and Seymour, 2011). Chemotherapy and radiotherapy are known to exacerbate the inflammatory state in patients, the adjuvant application of non-psychoactive cannabinoids such as, CBD and CBDA, may provide some vital symptomatic relief from the side effects of these therapies. One such side effect is gastrointestinal mucositis, which is commonly induced by radiotherapy (Ong *et al.*, 2010). The systemic inflammatory enhancements often produced by chemotherapeutics are a huge concern in oncology. Many of the molecular mechanisms targeted by chemotherapeutics result in an unwanted promotion of inflammation in cells which can ultimately lead to the failure of and resistance to, chemotherapy (Vyas, Laput

and Vyas, 2014). Similar issues are encountered with radiotherapy, tumour irradiation induces a wound healing response that is characterized by inflammation, cancer-associated fibroblast (CAF) modulation and extracellular matrix (ECM) remodelling, which may facilitate tumour recurrence (Barker *et al.*, 2015). Given this clear need for novel adjuvant immunotherapeutics, cannabinoids indeed offer an exciting novel therapy that warrants further exploration, not only as adjuvant therapeutics, but also for their clear anti-cancer potential.

In future studies it would be prudent to quantify the apoptotic potential of phytocannabinoids in conjunction with existing chemotherapeutics to see if adjuvant phytocannabinoid treatment may aid or outperform the efficacy of chemotherapy and radiotherapy (Ma *et al.*, 2011). In our studies we obtained inconclusive results when analysing the anti-metastatic effects of CBD and CBDA on VEGF. To better assess the anti-metastatic and anti-invasive properties of other cannabinoids, exploring their abilities to inhibit cancer progression and metastasis using explanted cancer cells. Migration assays would be utilised to gain a broader view of these treatments on the angiogenesis and metastasis of CRC. In these studies, we will assess the anti-metastatic abilities of phytocannabinoid by organ culturing of explanted CRC cells (Randall *et al*., 2011). Tissue culture of this kind can be maintained for a period of several days at a time whilst maintaining physiological features (Moorghen *et al*., 1996; Dame *et al.*, 2010). These cultured cells could be utilised in for a series of trans-well migration assays to observe the anti-metastatic potential of cannabinoids. In addition to this, the effects of an applied electric field could be used to monitor the electrotaxis of cancer cells, in healthy and cancerous

mucosa. Following this, the efficacy of cannabinoid treatments on the electrotaxis of cancer cells could be established. The high metastatic potential of CRC highlights the importance of exploring these effects in CRC (Tsujii *et al*., 1997).

To improve studies of this kind in future work, exploration into the wider physiological effects of cannabinoids must be considered. This may involve the collection of serum, saliva, and potentially urine samples from volunteers alongside tissue explants to extract more information regarding the metabolism of these drugs. Work such as this would aid in facilitating a wider appreciation of how elements of the ECS may be altered in cancer patients, providing a template for targeting particularly abnormal or altered ECS tone. In addition to this, modes of administration will need to be explored. Though crude systemic treatments achieved through oral administration or through inhalation, have achieved therapeutic effects. Further refinements in the administrative methods of cannabinoid therapies will lead to advancements in our ability to reduce the impact and occurrence of off target effects and sequelae, similar to as we have achieved with opiates.

4.5 Conclusions

Drugs that normalize the tumour microenvironment or interrupt the crosstalk between tumour and the surrounding microenvironment are an important approach to the management of cancer (Klampfer, 2011). In our studies we demonstrated the breadth of potential that micromolar ranges of CBD and CBDA have as anti-proliferative, cytotoxic and anti-inflammatory compounds in CRC. We

also highlighted the potential implication of the GPR-$_{55}$, 5-HT$_{1A}$ and PPAR$_{\delta}$ receptors in the mechanisms of action that underly these anti-cancer effects. Our findings contribute further towards the scientific understanding of the anti-tumoral effects of CBD and CBDA and support the continued exploration of these molecules as future cancer therapeutics. Our aim moving forward, is to continue translating existing *in vitro* knowledge into *ex vivo* human tissue to progress the application of these treatments clinically. Scientific insight into these molecules is significantly lacking. It should be made clear that, before advising patients medically, researchers and clinicians should develop an understanding of the tremendous medical significance of the chemicals employed. Practitioners would significantly benefit from developing an in depth understanding of cannabinoid molecules and their pathways, and in fact have a moral obligation to do so. This is a matter not only of scientific importance, but of great ethical importance. The onus is on clinicians and researchers to be able to provide patients with the highest standards of information and advice to ensure that patients are truly informed of all medical options available to them. Continued research into this field will enable researchers to develop a new field of medical treatments to aid medical professionals in continuing to deliver the highest quality of medical care.

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Karwad, M. A. *et al.* (2016) *The role of CB1 in intestinal permeability & inflammation Endocannabinoids control permeability and inflammation via CB1 2 List of Abbreviations AEA Anandamide 2-AG 2-Arachidonoyl glycerol NAPE N-acylphosphatidylethanolamine NAPE PLD N-acylphosphatidy*. Available at: http://eprints.nottingham.ac.uk/45054/1/FASEEB cb1 2nd submission.pdf (Accessed: 10 September 2018).

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6 Appendices

6.1 Appendix 1. Patient information sheet

Derby Hospitals NHS
NHS Foundation Trust

PATIENT INFORMATION SHEET

Derby City General Hospital
Uttoxeter Road
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DE22 3NE

Tel: 01332 340131
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Genetic and Vascular Studies of Colorectal Patients

You are invited to take part in a research study. It is important for you to understand why the research is being done and what it will involve. Please take time to read the following information carefully and discuss it with us if you wish. Please ask us if there is anything that is not clear or if you would like more information. Thank you for reading this.

What is the purpose of this study?

This study is in four parts. The first part involves looking at what changes in genes and protein expression occur in colorectal cancer within the primary tissues and in the blood. We hope to understand more about how these genes are controlled and how these control mechanisms are changed in the development of colorectal cancer. This would hopefully increase our knowledge of how and why these genes are involved in the development of colorectal cancer and may ultimately lead to targeted treatments and sensitive diagnostic tests. The second part of this study is to investigate a number of chemicals that might potentially cause blood vessels to relax, and so may help to control the circulatory system**. The third part is to examine how intestinal epithelial cells (cells that make up the inside lining of the bowel) react in the presence of inflammation and infection (for example appendicitis) and the chemicals produced.** Results collected from this study will contribute to dissertations for undergraduate and postgraduate degrees. **The fourth part of this study will examine the effects of**

cannabinoids on cancerous and inflamed colonic tissue. Previous research has demonstrated that these molecules have potential in treating an of colonic disorders, this study is the first of its kind to investigate the effects in humans. This research will increase our understanding of how these molecules interact within the human colon and demonstrate the potential of cannabinoids in colonic disorders.

Why Have I Been Chosen?

You have been chosen because you are to undergo colorectal surgery.

Do I Have To Take Part?

It is up to you whether you wish to take part or not. If you do decide to take part you will be asked to sign a consent form and be given this information sheet. You may decide to withdraw from the study at any time and you do not have to give us any reason for this. A decision not to take part or to withdraw from the study will NOT affect the standard of care you receive. Should you withdraw from the study your tissue sample and any data collected will be destroyed.

What Will Happen If I Decide to Take Part?

Once you are admitted to hospital you will have a blood sample taken for this study before your operation and whilst you are still in hospital after your operation.

During your operation some of your bowel is removed (this is a standard part of the operation). We propose to take a very small part of this removed tissue for laboratory analysis. The tissues will be destroyed after laboratory analysis, your details will be coded and none of your details will be available in results or publications. A small amount of demographic data such as date of birth and sex will be included in the trial master file only.

What Do I Have To Do?

If you wish to take part then we need your permission to collect these samples of tissue for later analysis in the laboratory. You can do this verbally and by signing the consent form.

Are there any side effects in taking part and are what are the possible disadvantages and risks in taking part?

No. We are not proposing to give you any new medication or drugs. No additional tissue will be removed – only that which would be removed during your surgery anyway. There will be no direct benefit to you but the results may help future treatments.

Are there any ethical issues?

All Research in the NHS is looked at by an independent group of people, called a Research Ethics Committee to protect your safety, rights, wellbeing and dignity. This study has been reviewed and given a favourable opinion by Derbyshire Research Ethics Committee.

What if I have any complaints?

If for any reason you wish to make a complaint about this study, you can contact the Patient Advice & Liaison Services at any time on 01332 785557.

What will happen to the results of the study

The results of this study will form part **of undergraduate and postgraduate students' dissertations** and will be published in National/International meetings and publications.

What if I have any questions?

We would be delighted to provide further information and answer any questions you might have. You may contact the research team.

Mr. Lund 01332 724704

Mr Couch 01332 724681

Mr Tasker 07904866250

You will be given a copy of this information sheet and your signed consent form for you to keep if you decide to take part in this study.

6.2 Appendix 2.

Centre Number:
Study Number:
Patient identification number for this trial:

CONSENT FORM

Title of Project: Genetic and Vascular Studies on Colorectal Patients

Chief Investigator : Mr. Jon Lund

Assistant Investigators: Dr. Saoirse O'Sullivan, Dr Daniel Couch, Dr. Cristina Tufarelli.

Please tick boxes

1	I confirm that I have read and understand the information sheet **version 10, dated 1** for the above study and have had the opportunity to ask questions.	☐
2	I understand that my participation is voluntary and that I am free to wit at any time, without giving any reason, without my medical care or legal rights being affected	☐
3	I understand that sections of any of my research records or medical notes where appropriate may be looked at by responsible individuals from the University of Nottingham or from regulatory authorities where it is relevant to my taking part in research. I give permission for these individuals to have access to my records.	☐
4	I agree to take part in the above study and approve the use of my tissue **and blood samples** in this study.	☐
5	I agree to my DNA, RNA **and proteins** being stored and I am happy for them to be in future research.	Yes* ☐ No* ☐

* Please delete

_____	_____	_____
Name of patient	date	signature
_____	_____	_____
Name of person taking consent	date	signature
_____	_____	_____

Researcher date signature