The effect of polymeric formula on enterocyte differentiation

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Abstract

Exclusive Enteral Nutrition (EEN) is commonly used in the management of Crohn’s disease (CD). Polymeric formulae (PF), comprising whole proteins along with the daily requirements of vitamins and minerals, are commonly used for EEN. The mechanism by which several weeks of PF treatment induces remission is incompletely understood and appears to be a combination of modulation of the intestinal microbiota and anti-inflammatory effects on the intestinal mucosa. These include a reduction in inflammatory cytokine production and improved barrier function.

The hypothesis for the current work was that PF caused an increased rate of differentiation of enterocytes at physiological concentrations. This was tested using Caco-2 cell culture and murine enteroids as in vitro models of the intestine.

When Caco-2 cells were treated with PF, it caused an increase in intestinal alkaline phosphatase (IAP) expression and activity, a marker of enterocyte differentiation. This was associated with a decrease in cell proliferation and viability. Murine enteroids dissociated after several hours of exposure to PF, possibly due to interference with Wnt signalling.

It was hypothesised that the observed effect of PF on Caco-2 cells might be mediated via the vitamin D receptor (VDR). However, when the activity of this receptor was reduced through use of an inhibitor, no change in PF-induced IAP activity and/or decrease in proliferation was observed. This suggests that the VDR pathway is not the primary driver of the accelerated differentiation seen in PF-treated cells.
Acknowledgement

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Chapter 1. Introduction

1.1 Crohn's disease

Crohn's disease (CD) is an inflammatory bowel disease (IBD) that can involve the whole gastrointestinal tract [1]. An over-reactive immune response results in patchy inflammation, leading to an altered intestinal environment as well as reduced digestive and absorption ability [2]. Symptoms flare up and get better again in periods of remission but it is a lifelong disease. Several environmental factors such as diet and antibiotics have been linked to the development of CD [3-5], that may contribute to an altered microbiota, considered another risk factor [5]. Chronic colonisation with pathogenic bacteria [6], and host genes associated with endoplasmic reticulum stress, anti-microbial peptide (AMP) secretion and autophagy [5] are also implicated. Thus, an as yet unknown combination of genetically predisposed enterocytes and colonocytes, environmental factors and/or the presence of microbial dysbiosis (that may also include the presence of pathogenic bacteria) are likely to increase the risk of relapsing disease through mechanisms that include inflammation and/or bacterial invasion.

1.2 Environmental factors

The environmental factors that have been identified in case-control and cohort studies are varied, differ between cohorts of different ethnicities, and the mechanism(s) involved are poorly understood. Being a smoker, undergoing an appendectomy, and not being breastfeed as an infant are associated with an increased risk [4]. Dietary factors are also implicated, and may include consumption of emulsifiers that cause disruption of the protective mucus layer [5]. Bile acids are endogenous emulsifiers, but are normally completely reabsorbed in the ileum, preventing disruption of the protective mucus layer in the colon where there are much higher concentrations of bacteria and the consequences of thinner mucus would be greater [7]. The cells found at the surface of intestinal crypts are mainly absorptive cells which remove water and salt from the mucus, causing it to dehydrate and thicken so that it is more difficult for bacteria to move through, protecting goblet cells in the crypt where the mucus is less viscous in order for it to be more easily secreted [7]. Stress interferes with mucus production and control of viscosity, providing a potential contributing explanation as to why stress can cause worsening of symptoms in CD [7].
There is also growing evidence that food additives such as maltodextrins, which are short glucose chains used to thicken food products, may increase expression of type 1 pili in AIEC, increasing its ability to adhere to host epithelium and form biofilms [8]. The recent increase in CD, especially in more industrialised nations [9] is likely to be at least in part due to these and other food additives more commonly consumed as part of a highly processed diet, over-zealous or unnecessary anti-biotic use [10] and possibly environments that don't facilitate healthy immune tolerance [11].

1.3 Microbiota

Reduced diversity within the gut microflora has been implicated in CD, as well as an overall reduction in bacterial abundance [5, 7, 12]. Significant changes to the representation of the major bacterial groups have also been reported, with a reduction in Firmicutes and Bacteroidetes, and an increase in mucosal adherent bacteria [5, 12].

CD patients reportedly have a greater proportion of *Escherichia coli* species within their gut microflora that include a subtype known as Adherent Invasive *E. coli* (AIEC) [13]. AIEC possess characteristic virulence factors that include expression of an altered type 1 pili FimH that enables bacterial adherence to the intestinal wall, resulting in upregulated expression of AIEC type 1 pili ligand CEACAM6 by the host cells [14]. Binding to CEACAM6 triggers AIEC internalisation by the enterocyte [15], and there is evidence that internalised bacteria can persist in susceptible individuals [16]. In addition, engagement of the CEACAM6 receptor by AIEC is associated with increased adherence of the enterocytes to the extracellular matrix (ECM). This prevents exfoliation, normally considered a process whereby infected cells are shed from the intestinal wall as an innate defence against infection [15].

Adhesion of AIEC to human cell lines is not CEACAM-exclusive [17]. Furthermore, the type 1 pili used by AIEC to CEACAM6 are not unique to these bacteria. The *E. coli* subtype K-12 carries the same type 1 pili but is not invasive [18]. CEACAM6 mediated internalisation by host cells is a kind of endocytosis whereby the cell microvilli elongate to envelop the bacteria, powered by the movement of microfilaments and microtubules of the cytoskeleton [17]. From here AIEC can cause lysis of the endocytic vacuoles, allowing the bacteria to escape into the cell cytoplasm [17]. Once the cell is infected, AIEC causes disruption of tight junction complexes resulting in increased intestinal permeability [19]. This is a predictor of relapse in quiescent CD [20]. Once past the epithelial cell layer AIEC have the ability to survive and multiply in human macrophage
phagosomes, causing chronic tumour necrosis factor (TNF)-α secretion that further propagates the harmful immune response [16]. These characteristics make AIEC capable of driving chronic granulomatous inflammation, characteristic of CD lesions [1].

Patients with CD are also shown to have an increased immune reactivity to bacterial epitopes, including those of commensal bacteria, which may drive uncontrolled inflammatory reaction [5]. This may, in part, reflect the presence of specialised immune cells called microfold or M cells that sit between epithelial cells lining the intestine. The Peyer’s patches of the ileum are especially concentrated with these cells and here there is also a reduced mucus barrier to facilitate exposure to non-invasive bacteria [6]. Normally, M cells help build tolerance of the epithelial cells to normal microflora antigens [6]. However, in patients with CD, this environment reportedly exacerbates bacterial biofilm formation on enterocytes in the ileum, contributing to this location being one of the most common sites of inflammation.

1.4 Genetics
Genome wide association studies have identified at least 71 gene loci that are associated with an increased risk of CD [21]. The three most strongly associated are autophagy related protein 16-like 1 (ATG16L1), immunity related GTPase family M (IGRM) and nucleotide oligomerization domain 2 (NOD2). ATG16L1 normally forms a protein complex that allows formation of autophagosomes that enable cells to degrade old or dysfunctional organelles, consume cellular components for energy in times of starvation, and are also important for killing intracellular pathogens [21]. The exact action of IGRM is not fully known, but is also thought to be involved in autophagy [21]. Mutated alleles of NOD2, that normally codes for an intracellular receptor for bacterial muramyl dipeptide prevents Paneth cells and immune cells from detecting and thus responding to infection as effectively [21]. NOD2 mutations are also associated with leakier intestinal epithelium, both in CD patients and 10-30% of their first degree relatives [22]. Another risk gene Xbp1 is involved in endoplasmic reticulum stress response and AMP secretion by Paneth cells, reducing the ability of the host cells to cope with the stress of infection, and further decreasing AMP secretion [5]. Patients with CD have been shown to secrete less AMPs even in times of remission [5]. The greater the number of at risk alleles, the higher the risk of developing disease via the creation of an environment in which bacteria can more easily pass through the
protective mucus and infect the host cells that, in turn, have a reduced ability to clear the infection [5].

1.5 Inflammation

The symptoms of Crohn’s disease are fuelled by recurring bouts of uncontrolled gastrointestinal inflammation. Patients with CD have been found to have an overactive immune response against even normal bacterial antigens, for which healthy individuals have a tolerance, suggesting that they have a pro-inflammatory predisposition [6]. TNF-α and other inflammatory mediators have been shown to promote inflammation in the gut and increase leakiness of the intestinal wall through increasing activity of myosin light chain kinase (MLCK), which causes disorganisation of tight junctions [6]. This allows luminal bacteria access to the gut mucosa where innate defences further promote inflammation [23]. The pathogenesis of CD has been linked to constitutive overproduction of TNF-α, produced by chronic activation of leukocytes, In addition to inflammation, TNF-α also increases apoptosis in differentiated intestinal cells, causing death of the cells that provide the best barrier to bacterial infection [7], evidenced by the therapeutic success of anti-TNF-α antibodies in patients with CD [23].

1.6 Epithelial barrier function

The lining of the gastrointestinal tract is exposed to anything that is ingested, having to simultaneously allow absorption of macro and micronutrients while preventing anything potentially harmful from entering the bloodstream [24]. In order to perform both these functions in the small intestine a wall of enterocytes carry enzymes, regulated transporter proteins and protein complexes that hold the cells together in a tight layer [25]. Tight junctions at the apical surface restrict the movement of water and solutes and are essential for barrier integrity [25]. Dysfunction can be caused by inflammation, with the consequence that bacteria and their by-products can more easily enter the mucosa and submucosa, and trigger further inflammation [22].

Enterocytes have a high rate of cell turnover. They are produced by stem cells in intestinal crypts and migrate up the villus over 4 to 8 days, and then are shed at the apex of the villus (Figure 1.1) [7, 12]. These stem cells also produce enteroendocrine cells which release hormones in response to luminal contents, goblet cells which produce the protective mucus layer, and Paneth cells that move down the crypt and protect the stem cells by releasing antimicrobial peptides (AMPs) [26].
Figure 1.1: Small intestine cell differentiation and migration. Stem cells that reside in the crypt generate enterocytes, endocrine cells, and goblet cells that migrate up the villus where they die and are shed by anoikis. Paneth cells are also produced; these cells migrate to the base of the crypt where they protect the stem cells through the production of anti-microbial peptides. Sourced from [12].

In healthy individuals, bacteria are preventing from coming into contact with the intestinal wall by a layer of mucus [7] that, in addition to forming a barrier, also contains secreted IgA specific to bacterial epitopes that help to further restricting bacterial movement [6]. However, biopsies from patients with CD show a concentrated layer of bacteria growing directly beside the enterocytes, with some even surviving within the cells [7].

Anti-microbial peptides (AMPs) are small proteins with broad anti-pathogen effects, and include defensins, cathelicidin, lysozyme, and phospholipase A2, all of which are produced in the small intestine primarily by Paneth cells. In addition, the enterocytes that form the gut barrier secrete alkaline phosphatase (ALP) [27, 28]. ALP is an enzyme thought to enact an antibacterial effect through dephosphorylation of pro-inflammatory lipopolysaccharide [29]. However, evidence that ALP is produced in
increasing quantities as enterocytes differentiate has led to ALP secretion being used as a marker of enterocyte differentiation (that includes both transformed and primary cells) [28, 30, 31].

Reduced bacterial counts are typically seen in more inflamed regions of the intestine due to the migration of stimulated white blood cells into the mucus layer, but the bacteria that are taken up by cells can cause ongoing inflammation, potentially leading to ulcers and abscesses [7]. Interestingly, infected enterocytes are more often seen at the bottom of the intestinal crypts, where they are generated from a pool of stem cells. As these cells migrate from the crypt to the villous they develop a more robust phenotype as a result of differentiation, with fully differentiated cells reportedly less likely to be infected despite a much greater exposure to bacteria [7]. Thus cell differentiation appears to provide a more effective barrier to further bacterial invasion and inflammation. A well differentiated epithelium, the overlying layer of thick mucus and the anti-microbial peptides that it contains all help prevent the movement of infectious or toxic agents from entering the body.

1.7 Vitamin D

Active vitamin D, otherwise known as vitamin D3 and as 1,25, dihydroxycholecalciferol, is a hormone that plays an important role in regulating Ca$^{2+}$ and PO$_4^{3-}$ absorption, storage and excretion, as well as immuno-modulatory effects [32]. It is estimated that around 3% of the human genome is regulated directly or indirectly by the vitamin D endocrine system [32]. Vitamin D is synthesised in vivo with exposure to ultraviolet-B radiation (UVB), which converts a cholesterol derivative to cholecalciferol that is released from the skin into the blood stream. Cholecalciferol is converted to the prohormone calcidiol in the liver, which is then transported in the blood by the Vitamin D Binding Protein. An enzyme found mainly in the kidneys called CYP27B1 (25-hydroxyvitamin D$_3$-1α-hydroxylase) converts the prohormone to the active form of vitamin D, calcitriol. However, evidence is emerging that this final step in this pathway can occur in other cell types that express this enzyme [33]. This includes monocytes, macrophages [34], enterocytes and colonocytes [35]. The vitamin D receptor (VDR) is a nuclear receptor that dimerises with the retinoid-X receptor in the presence of calcitriol, allowing it to bind to the hormone response elements that control over the expression of certain genes [30]. Accordingly, VDR mediates most of the known downstream effects of active vitamin D [33].
Recent evidence has found that long term vitamin D supplementation improves symptoms for people with CD [36]. The addition of vitamin D to an intestinal cell line causes resistance to the effects of TNF-α, with improvements in monolayer leakiness, tight junction organisation and reduction in inflammatory transcription factor Nuclear Factor (NF)-κB activation [20, 22, 32]. Vitamin D up-regulates expression of several tight junction proteins, including occludin, ZO-1, ZO-2, and vinculin [37]. Vitamin D has been shown to induce the expression of intestinal alkaline phosphatase (IAP) [30] and cathelicidin [34], two anti-microbial peptides that are also markers of enterocyte differentiation [38]. There is also evidence that activation of the Vitamin D receptor can affect the Wnt signalling pathway via decreased β-catenin and increased E-cadherin activity, resulting in decreased cell division and increased differentiation in cultured cells [37].

1.8 Butyrate

Butyrate is a short chain fatty acid produced by bacteria in the colon from carbohydrates that cannot be broken down by digestive enzymes in the small intestine. Normally considered to provide an important energy source for colonocytes, there is growing evidence that butyrate, like Vitamin D, can affect host cellular pathways.

Exposure to butyrate causes an increase in expression of a vitamin D activating enzyme [33] and is associated with a dose-dependent increase in IAP activity, CEA protein concentration and trans-epithelial electrical resistance (TEER) [39]. Butyrate is also shown to inhibit Caco-2 cells from moving into the DNA synthesis (S) phase of the cell cycle without increasing the rate of cell death, which appears to mimic spontaneous differentiation [39]. This butyrate-related effect of enhanced cell differentiation most likely helps maintain barrier integrity against inflammation-perpetuating bacteria, and provide a possible indirect mechanism for the benefits of PF. Intriguingly, butyrate promotes cell differentiation and apoptosis in cancerous cells and cell lines while having the opposite effect in healthy colonocytes [30]. These differences may be explained by a much slower butyrate metabolism demonstrated in Caco-2 cells that may cause butyrate to accumulate within cells and elicit the toxic and pro-apoptotic effects at much lower concentrations [39]. Normal cells would also have the protection of a layer of mucus that would insulate the cells from a high luminal concentration [39]. Thus effects in cell lines derived from abnormal cells may differ from those seen in vivo but butyrate still provides an alternative stimulant and positive control for many markers of cell differentiation.
1.9 Enteral nutrition

As CD can cause loss of appetite and severe malnourishment, and may be resistant to standard treatment, patients may be given exclusive enteral nutrition (EEN), a nutritionally complete liquid diet. EEN has been shown to have effects in the body beyond mere nutrition, especially when given exclusively over many weeks, and is just as effective as corticosteroids and infliximab at inducing remission [23], but without the side effects that can mean that they are contraindicated or intolerable. For infliximab these include increased susceptibility to common infections, risk of lymphoma and production of anti-infliximab antibodies that render the drug ineffective [9]. Long term use of corticosteroids commonly causes poor wound healing, central adiposity, loss of bone density, immunosuppression, as well as growth retardation in children [9]. Some antibiotics can cause short term improvement in CD, but they are not an effective long term treatment and have side effects as a consequence of their lack of specificity, affecting both harmful and helpful bacterial species [40].

There are two main kinds of formulae that may be used for EEN: elemental and polymeric, which means that they are composed of predominantly simple sugars and amino acids, or whole carbohydrates and proteins, respectively. Polymeric formula (PF) is cheaper, more palatable, and is just as effective as elemental formula despite requiring additional digestion [41]. Accordingly, PF is increasingly being used to treat children and adults with CD.

PF causes changes in the microflora, favouring Bacteroides species that have been associated with a reduction in inflammation [42]. PF inhibits the degradation of IκBα, resulting in decreased NF-κB activity, a transcription factor that promotes an inflammatory response and TNF-α production [43]. PF increases the expression of carcinoembryonic antigen-related cell adhesion molecule (CEACAM) 6, a marker of enterocyte differentiation [44] and prevents TNF-α induced tight junction dysfunction, IL-8 production, and increase in epithelial permeability at concentrations of 10% and 20% which would be found in someone taking PF exclusively [23]. However the mechanism(s) behind its effectiveness in treating CD are not yet fully understood.

Vitamin D is included in standard PF preparations in order to ensure that all vitamin recommended intakes are met enterally, even though most individuals get most of their vitamin D through skin exposure to UV radiation. Therefore in CD patients on long
term EEN, levels of intra-luminal vitamin D are likely to be much greater than would otherwise be present.

1.10 Research aim

The mechanism by which immunosuppressants treat IBD are relatively well understood. However, currently used dietary treatments contain a complex composition of potentially active agents, which makes it more difficult to elucidate how they afford protection. Polymeric formula has been shown to have many different effects on both enterocyte function and microbiota composition, but the underlying mechanism(s) for each of these has yet to be demonstrated. There is evidence that dietary components can affect the differentiation of the intestinal epithelium [30, 39]. Differentiation has been shown to improve barrier function, dysfunction of which is a characteristic of those who have, and those who are at increased risk of CD [19, 25].

The aim of this project was to investigate the effects of polymeric formula as used for EEN on the rate of intestinal epithelial cell differentiation, using two models of the small intestine. The models to be used include a continuous enterocyte cell line and three dimensional primary cell culture. The intention of this project is to increase understanding of how polymeric formula is effective at managing CD, in order to improve outcomes by better uncoupling the viscous cycle of inflammation that drives it.
Chapter 2. Materials and Methods

2.1 Caco-2 cell culture
Caco-2 monolayers were incubated in Dulbecco's DMEM (Gibco, Auckland, New Zealand) with 10% Fetal Bovine Serum (Gibco), 1% Penicillin-Streptomycin (Gibco) and 1% Non-Essential Amino Acids (Gibco) at 37°C under 5% CO₂ in 75mL flasks. Passages 56-95 were used for all experiments. Cells were passaged weekly, with TrypLE Express (Gibco) used to lift the cells. Cell monolayers cultured for more than 7 days had their medium changed every two or three days after the first week. For experiments, Caco-2 cells were cultured in twenty-four well plates with a well area of 2cm² (Nunc, Sigma-Aldrich, New Zealand) under 1mL of medium, or 12 well plates (Nunc, Sigma-Aldrich, Auckland, New Zealand) with an area of 4cm² under 2mL of medium. Incubation time with TrypLE Express was increased from 10 to 20 minutes to reduce clumping of the cells, which was found to be an issue especially as cell density increased. Also, cells treated with PF required additional washes in PBS to remove as much residue as possible before TrypLE Express was added. This enabled cells to be lifted.

2.2 Cell counting
To count the cells, 20µL of cell suspension was diluted 1:1 in 0.5% (w/v) trypan blue (Sigma-Aldrich) in PBS, mixed and pipetted into a haemocytometer. The number of cells in the 16 square grids in each corner were counted, averaged, doubled (to take into account dilution in trypan blue) and multiplied by 10⁴ to give the concentration of cells per ml of suspension. Non-viable cells are permeable to trypan blue dye and appear blue under the microscope, enabling estimation of viable versus non-viable cell numbers.

2.3 Flow cytometry
Caco-2 cells (5 x 10⁴ or 1 x 10⁵) were cultured in twenty-four well plates for 3 to 7 days. The cells were washed twice with 0.5mL PBS, then incubated at 37°C for 10 minutes with 300µL TrypLE Express (Gibco). Three hundred microliters of medium was added to each well and the resultant cell suspension centrifuged at 800 x g for 3 minutes. The speed at which the cells were pelleted was initially 500 x g but this was increased to 800 x g, as this did not affect cell viability while creating a tighter pellet that was less likely to fall apart when the supernatant was aspirated. After aspiration the cells were
re-suspended in 300µL medium. This was divided between two V-bottomed wells in a 96 well plate (Nunc, Sigma-Aldrich) (130µL each), and then centrifuged at 295 x g for 5 minutes. Both supernatants were aspirated and one pellet was re-suspended in 50µL PBS, the other in 50µL Annexin Binding buffer (Invitrogen, Auckland, New Zealand). Five microlitres of propidium iodide (Invitrogen, Thermo Fisher Scientific, Auckland, New Zealand), and 2.5µL of Annexin V fluorescein conjugate (Life Technologies, Thermo Fisher, Auckland, New Zealand) was added to each sample, which were incubated at RT in darkness for 15 minutes. A further 150µL of either PBS or Annexin Binding buffer was added to the respective samples before reading by the cytometer. The PBS Annexin control was used to set the threshold for FITC auto-fluorescence. The proportion of cells over this threshold were considered apoptotic or dead depending on whether they were PI negative or positive respectively.

2.4 Intestinal alkaline phosphatase activity

Intestinal alkaline phosphatase (IAP) activity was determined by measuring the change in absorbance as the colourless substrate para-nitrophenylphosphate (PNPP) was hydrolysed to form the yellow product para-nitrophenol (PNP) [45]. The substrate solution (2.5g/L PNPP (Sigma-Aldrich), 100mM diethanolamine, 150mM NaCl, 2mM MgCl₂, pH 9.5) was aliquoted and stored at 20°C until required to reduce the rate of spontaneous decomposition. The collection buffer (0.1M Tris-HCl, 150mM NaCl, pH 8.0) and was kept at 4°C.

Caco-2 cells were washed once with 1mL PBS before the addition of 300µL of cold collection buffer to each well. The reverse end of a 200µL pipette tip was used to scrape the bottom of the well, with the resultant suspension of fragmented cells collected into a microfuge tube and mixed vigorously by pipetting to aid further dispersion. Fifty microliters of cell lysate was added to each of 2 microcentrifuge tubes, and 150µL of pNPP substrate solution was added to one of each after set time intervals. To the other microcentrifuge tube, 150µL of substrate solution not containing PNP, followed by 50µL of 0.5M NaOH was added as a blank for each sample. Samples were then repeatedly inverted to prevent the cells and cell fragments from settling out. After 8 minutes, 50µL 0.5M NaOH was added to the test samples to stop the reaction. The tubes were centrifuged for 3 min at 4,000 x g to pellet the cell debris. Two hundred microliters of supernatant was removed and added to separate wells in a 96 well plate. Absorbance was read at 405nm using a Spectramax-190 platereader (Molecular Devices) and Softmax Pro V5.3 software (Molecular Devices).
The relationship between the concentration of para-nitrophenol (PNP) and its absorbance at 405nm at the same volume and pH as present in the enzyme assay was determined to allow the results to be converted from abstract absorbances to units of concentration. Multiple concentrations of the PNP product in the linear range of absorbance were averaged across three independent measurements and the gradient used to determine the constant (Figure 2.1). Dividing the absorbance by 0.028 gave the concentration of PNP product in µM.

![Figure 2.1 PNP concentration versus optical density](image)

**Figure 2.1 PNP concentration versus optical density.** Optical densities at 405nm (OD405) were graphed against the concentration of PNP (µML), and the resultant standard curve used to enumerate IAP activity in samples by absorbance. The multiplication factor derived from the gradient was 0.028. Results shown are ± SEM of 7 independent experiments.

All IAP measurements were performed on live cells as freezing was found to decrease activity (results not shown). All samples at each time point were exposed to the same substrate solution at the same temperature over the same period of time to allow comparison of IAP activity. To determine the amount of PNP in cell homogenates in µM, absorbances were multiplied by 3 (50µL from 150µL) and divided by 0.028, the value derived from the gradient of the graph (Figure 2.1). IAP activity in the cell culture supernatant was determined by multiplying absorbance by 20 (50µL from 1mL) and dividing by 0.028.

Each assay included samples from duplicate wells. The volume of sample, PNPP substrate concentration, incubation time and temperature for the IAP assay were adjusted to ensure that the levels of IAP activity in the samples fell within the linear range of change in absorbance over time. Due to the opacity of the cell medium with the addition of 20% (v/v) PF cell medium, it was not possible to measure IAP activity in the supernatant of these samples.
2.5 Western Blotting

Caco-2 cells (1 x 10^5) were grown in 24-well plates for 6 to 8 days (with and without the addition of 20% (v/v) PF at day 5). Media was removed and the cells were washed once with PBS before being lifted with TrypLE and pelleted, as described previously. The pellet was re-suspended in 100µL of RIPA buffer (50mM Tris-HCl pH 7.5, 150mM NaCl, 1% (v/v) NP-40, 1x protease inhibitor (Complete Mini; Roche, Basel, Switzerland), 2% SDS, 2% mercaptoethanol, 5% glycerol v/v, 0.01% (w/v) bromophenol blue), transferred to a microcentrifuge tube and kept on ice for 30 minutes before centrifugation for 20 minutes at 30,000 x g. The resultant cell lysate pellet and supernatant were separated and frozen at -20°C.

Protein concentration was initially measured using a Direct Detect Infared Spectrometer (Merck Millipore, New Zealand). However, residual PF in some cells lysates meant that cellular protein concentration could not be used to control for sample loading onto gels. Instead, a fixed volume of each sample that had been prepared the same way was used, with densitometric analysis of the IAP band compared to that of GAPDH (as a housekeeping gene) used to determine the relative difference of IAP expression between the different conditions.

The samples were diluted 1:1 in PBS and then 4:1 with 5x sample lysis buffer (300mM Tris, 10% (w/v) SDS, 25% (v/v) glycerol, 0.05% (w/v) bromophenol blue, 1.6M β-mercaptoethanol, pH 6.8) before being heated at 95°C for 10 minutes. A total of 15µL (sample and lysis buffer) was loaded onto a 12% polyacrylamide gel, with Precision Plus Protein Standards (Bio-Rad, Auckland, New Zealand) included on each gel as molecular weight markers. The gel was electrophoresed at 200V until the dye front reached the bottom of the gel (50-55 minutes). The separated proteins were then transferred to a polyvinylidene fluoride (PVDF) membrane (Hybond-P™, pore size 0.45µm; GE Healthcare, Little Chalfont, UK) using a wet transfer method, whereby the polyacrylamide gel and PVDF were sandwiched between filter paper in cold transfer buffer (10% (v/v) methanol, 50mM Tris-base, 0.38M glycine) under 100V for 60 minutes.

The PVDF membrane was blocked in 5% (w/v) bovine serum albumin (BSA; AlbuMAX I Lipid rich BSA, Gibco) in Tris Buffered Saline with 0.05% Tween 20 (TBST) for one
hour at RT before being incubated overnight at 4°C in primary antibody. Mouse monoclonal anti-IAP (Abcam ab54776, Melbourne, Australia) and rabbit polyclonal anti-IAP (Abcam ab95462) were used at a concentration of 1:500, diluted in 2% (w/v) BSA (in TBST). The membrane was washed four times for 5 minutes in TBST before incubation with the secondary antibody in TBST for two hours at RT. Goat anti-mouse IgG antibody conjugated to horseradish peroxidase (HRP) (Pierce 31430, Thermo Fisher Scientific) was used at a concentration of 1:5000, also diluted in 2% (w/v) BSA in TBST. Goat anti-rabbit IgG HRP conjugated antibody (Abcam, ab97051) was used at a concentration of 1:2000. The membrane was washed four times for 10 minutes each in TBST before being drained and incubated for four minutes under ECL Plus Pierce Blotting Substrate (Thermo Fisher Scientific). Images were created using UVITEC Cambridge Gel Documentation system (UVITEC, Cambridge, UK). Densitometry using UVIband software (UVITEC) was used to quantify the relative amounts of proteins in the bands.

2.6 Fluorescence microscopy

Caco-2 cells (1 x 10^5) were grown on 16mm round coverslips that were ethanol-sterilized before being placed in the bottom of each 4cm² well (in a 12-well plate) in total volume of 2 mL media per well. The cells were allowed to settle and grow for 5 days before the media was changed, with and without the addition of 20% (v/v) PF for up to 72 hours. The media was removed and the cells were fixed with the addition of 1 mL 4% w/v paraformaldehyde (PFA) pH 7.4 for 45 minutes at room temperature. The PFA was aspirated and the coverslips stored in PBS at 4°C until needed.

Before staining the cells, each coverslip was washed twice (total 20 minutes) in 2 mL 1,4-piperazinediethanesulfonic acid (PIPES) buffer (60mM PIPES pH 6.8) before unbound sites were blocked with 500µL of 5% (w/v) BSA in PIPES buffer) for 45 minutes at room temperature. Each coverslip was washed three times for five minutes in 2 mL PIPES buffer on a shaker. Enteroid cells were permeabilised with 500µL Triton solution (0.5% (v/v) Triton-X100 in PIPES buffer) for 15 minutes at room temperature before staining. Caco-2 monolayers were not permeabilised. After another two 5 minutes washes each coverslip was incubated overnight at 4°C on a rocker in 500µL of primary antibody (diluted in 2.5% (w/v) BSA in PIPES buffer).

Following four 5 minute washes in 2 mL PIPES buffer on a shaker, the membrane incubated in 500µL of FITC-conjugated secondary antibody (diluted in 2.5% (w/v) BSA
in PIPES buffer) for 2 hours at room temperature. 1:1000 Hoechst 33342 (Life Technologies; 10mg/L diluted in PBS) was added to each well for 20 minutes at room temperature to stain the nuclei. This was followed by four washes of 2 mL 0.05% (v/v) Tween-20 in PIPES buffer on the shaker, and a last wash for 10 minutes in PIPES buffer alone. The coverslips were removed from the wells and carefully touched to a paper towel to drain, then placed up-side-down on a slide with a drop of Antifade Mountant (0.1% w/v p-phenylenediamine, 50mM Tris-HCl pH 6.8). The slides were stored at 4°C in the dark until viewed under fluorescence microscopy. Images were taken using a Zeiss Axio Imager Z1 microscope (Auckland, New Zealand) with AxioVision software (Zeiss).

2.7 Cell migration assay

Half a centimetre was cut off the wider end of 200µL pipette tips, giving plastic collars that were then sterilised in 70% ethanol for 15 minutes. Excess ethanol was removed and each collar was placed (with the un-cut edge down) in the centre of a well (using a 12 well plate). The plate was then carefully placed in a 37°C incubator with the lid propped up slightly for at least 15 minutes. This allowed the ethanol and most of the water to evaporate, and facilitated adherence of the rings to the surface of the well. Caco-2 cells (1 x 10^5) in approximately 70µL of media were added to the inside of the ring, followed by another 50µL of medium to remove cells adhering to the sides of the ring. This resulted in a confluent layer of cells on the bottom of the well inside the collar following incubation for 24 hours at 37°C. Importantly, there was no evidence of cells and/or medium outside the collar after this time.

Medium with or without the addition of PF was added to the outside of the ring, which was removed leaving a circle of confluent cells centred in the middle of well. The cells were cultured for up to 18 days, at which timepoint untreated cells were shown to spread more than half the way to the edge of the well. A scalpel blade was used to mark the furthest point that the cells had reached in 2 dimensions, and then a ruler used to measure the distance to the nearest half-milimeter. From this distance, the initial diameter of 6mm was subtracted to give the change in length, and then the mean calculated for each treatment condition.
Figure 2.2: Cell migration assay. Wells on the left show the cell suspension trapped within the base of a pipette tip, whereas those on the right show the incubation phase where the medium has been added and the tips removed, allowing the cells to spread.

2.8 Enteroids

Juvenile male C57/BL6 mice were euthanized by neck dislocation. The small intestines were dissected out and flushed twice with ice cold PBS. The intestine was opened lengthwise and cut into 1cm pieces that were added to 5mM EDTA for 5 minutes on ice. The tissue was then transferred into cold PBS and shaken for 30 seconds, before being returned to the EDTA for another 5 minutes. This was repeated several times, with each fraction checked microscopically for quantity and proportion of villi and crypts.

The crypt-rich fractions were put through a 70µm filter to enrich for crypts, which were then pelleted by centrifugation at 600 x g for 3 minutes. The pellet was resuspended in 200µL of Matrigel Matrix (Corning, USA) that had been removed from the -20°C freezer and kept on ice. The resultant suspension was transferred to 12 or 24 well plates as a shallow bubble in the centre of the well. If intending to immuno-stain the enteroids, the Matrigel was laid down on sterile coverslips in the wells.

The Matrigel was allowed to set (10 minutes at 37°C) before culture medium (Advanced DMEM (Gibco)) with growth factors (100ng/L Murine Noggin (PeproTech, USA), 500ng/L Human R-spondin (PeproTech, USA), 50ng/L EGF (PeproTech, USA), 1mmol/L N-Acetyl-L-Cysteine (Sigma), 1x N2 (Gibco, USA), B27 (Gibco, USA) and 1x Penicillin-Streptomycin (Gibco) was added to just cover the top of the Matrigel [46, 47]. BMP antagonist Noggin, EGF and stem cell Wnt signalling agonist R-Spondin were
added again to the medium at the same concentration 48 hours later to promote growth and differentiation [47]. After two more days the existing medium was removed and new DMEM with fresh growth factors were added. This cycle of alternating full medium change and growth factor addition was repeated every two days over the course of the experiment.

A modification of this mode; involved incubating the stem cell rich crypts on a layer of Cellmatrix Type I-A porcine tendon collagen (Component A; Nitta Gelatin Inc., Osaka, Japan) [48].

Approval was received from the University of Otago, Christchurch Animal Ethics Committee to retrieve bowel tissue from animals being euthanized for another approved research/teaching protocol (Protocol GC2/14).

2.9 Statistical analysis

Results are presented as the mean ± standard error of the mean (SEM) for independent experiments repeated at least three times. All statistical analysis was performed using GraphPad Prism version 6.00 (2012) (GraphPad Software Incorporated, La Jolla, CA, USA). A t-test was used to compare differences between two groups of data, whereas one-way or two-way ANOVA were used for analysis of multiple data sets. Tukey’s test for multiple comparisons was used to compare data between more than two groups.
Chapter 3. Results

The transition of undifferentiated to differentiated epithelial cells is an important part of maintaining barrier function in the gut [39]. Differentiated enterocytes are less likely to become infected with invasive bacteria or become leaky to bacterial antigens in the presence of host inflammation [7, 23]. Thus an ability to increase the rate of epithelial cell differentiation was considered to be a potentially underappreciated benefit of PF treatment. In support of this hypothesis is evidence that treatment of cultured gut epithelial cell lines with PF is associated with increased expression of antimicrobial peptides [24], increased trans epithelial electrical resistance (as a marker of increased barrier function) [23], and increased expression of CEACAM6 (as a releasable decoy that limits bacterial adherence to the gut wall) [44].

The Caco-2 cell line was used as a model to investigate whether polymeric formula (PF) might promote renewal of the gut epithelium. Caco-2 cells were originally derived from a human colon adenocarcinoma but are widely used as a model of the human small intestine as the cells are easily grown in the laboratory and share many of the characteristics of enterocytes [49]. These include the formation of a tight monolayer that can restrict diffusion of solutes, development of a brush border of microvilli with functional digestive enzymes, and the production of anti-microbial peptides (AMPs) [39]. Osmolite (Abbot Nutrition, New Zealand), a standard polymeric formula, was added to Caco-2 cells and the rate of cell growth was compared to that of untreated cells. Twenty percent PF was used for these experiments as there is evidence to suggest that this is likely to be the intraluminal concentration that would be found in someone who was taking polymeric formula exclusively [23].

Intestinal alkaline phosphatase (IAP) is an AMP that is produced in increasing amounts as gut epithelial cells differentiate [24]. The IAP enzyme is expressed on the cell membrane before being released into the lumen where it dephosphorylates and inactivates the inflammatory bacterial lipopolysaccharide (LPS) [29]. IAP is commonly used as a marker of cell differentiation, and activity can be measured using a phosphatase enzyme assay [28, 30]. Accordingly, IAP expression and release were measured as a marker of Caco-2 cell differentiation.
The objectives for this chapter were to:

1. Investigate baseline measurements of untreated cells to determine cell numbers and time points for the proposed experiments.
2. To confirm the relationship between cell differentiation and IAP activity.
3. To determine the effect that co-culture with 20% (v/v) PF has on the rate of cell growth and IAP activity.

3.1 Cell density

Caco-2 cells seeded at two densities (1 x 10^5 and 5 x 10^4) increased in cell number over 7 days of culture. Not unexpectedly, the higher concentration of cells at T0 transitioned from a proliferative phase to reach a plateau of around 5x10^5 cells/well by day 5 whereas the lower initial seeding concentration of 5x10^4 cells/well required 6 days to reach a similar plateau (Figure 3.1). At this point the cells had reached confluence, and the genes involved in cell proliferation were likely down regulated in response to contact inhibition as the result of cell-cell contact [50]. For both starting cell concentrations, there was a steady increase in cell number over time, and at all subsequent time points cell numbers were significantly higher than observed at day 3 (P<0.0001).

![Figure 3.1: Caco-2 cell numbers increase over time.](image)

*Figure 3.1: Caco-2 cell numbers increase over time.* Trypan blue was added to Caco-2 cells before counting by haemocytometer. Results are the mean ± SE of three independent experiments. Raw data were analysed by 2 way repeated measures ANOVA. No significant differences were detected between the two concentrations of cells at any specific time point.
3.2 Cell viability

The percentage of non-viable cells (determined by uptake of trypan blue dye) did not show any clear trend over time (Figure 3.2A), a finding that was supported by flow cytometry of propidium iodide (PI) and annexin stained cells (Figure 3.2B). One difference noted between the two methods was that cell death rates were higher when cells seeded at the higher concentration were counted manually. Despite this slight difference, both methods indicated that the rate of cell death in untreated Caco-2 cells ranged from 5% to 10% over the course of the 7 day experiment.

![Figure 3.2: Assessing cell death.](image)

3.3 Intestinal alkaline phosphatase activity

Cell membrane-bound IAP activity increased each day (Figure 3.3A). Some of the initial increase was considered attributable to an increase in cell number, but as cell number began to stabilise IAP activity continued to rise. No difference in IAP activity was observed between the two different seeding concentrations of Caco-2 cells, with the average activity at day 3 between 9 and 10 micromoles of PNP per well, and between 25 and 27 micromoles by day 7. Over time the increase in IAP activity was significant ($P=0.005$) with both cell concentrations.

IAP activity in the cell supernatant also increased over the 7 days ($P=0.04$; Figure 3.3B), irrespective of cell seeding concentration. At day 3, an average of 64 micromoles of IAP was detected in the wells seeded with the higher concentration of cell ($1 \times 10^5$), whereas cells seeded at the lower concentration ($5 \times 10^4$) produced 77
micromoles per well. By day 7, levels of IAP activity had increased to 89 and 100 micromoles, respectively.

The rate of increase in IAP was greater in the cell supernatant than in the cell homogenates, which supports the hypothesis that IAP is secreted by the cell [29].

![Figure 3.3](image-url) **Figure 3.3: Intestinal Alkaline Phosphate (IAP) activity.** IAP activity was measured in (A) Caco-2 cell homogenate and (B) supernatant recovered following cell culture. Results are the mean ± SE of the independent experiments. Raw data were analysed by 2 way repeated measures ANOVA. No significant differences in IAP activity were detected between the two concentrations of cells at any specific time point.

### 3.4 Polymeric formula causes a decrease in cell number

The addition of 20% (v/v) PF to Caco-2 cells at day 5 of culture was found to have a marked effect on cell number over time (Figure 3.4). Untreated cells continued to proliferate over time and numbers were significantly increased at 72 hours ($P<0.01$). In contrast, there was no significant increase in cell numbers in the PF-treated wells. Consequently, cell numbers were significantly higher in control wells at 48 and 72 hours.
3.5 Polymeric formula is associated with loss of cell viability

The difference in Caco-2 cell density following treatment with 20% PF must be due to either a decrease in cell proliferation and/or an increase in rates of cell death. To explore this further, the rate of cell death in the same samples was monitored by determining the number of non-viable cells (assessed by trypan blue staining). The results of these experiments showed that cell death in the untreated cells ranged from 6% to 8% and did not increase over time. However, as shown in Figure 3.5, treatment of the cells with 20% PF caused a significant increase in cell death over time ($P<0.05$). Consequently, cell death was significantly higher in PF-treated wells at 48 and 72 hours.

Figure 3.4: Cell proliferation slows with the addition of polymeric formula. PF (20%) was added to day 5 cultures of Caco-2 cells, and cell numbers were counted over 72 hours. Results are the mean ± SE of eight independent experiments. Raw data were analysed by 2 way repeated measures ANOVA. Asterisks indicate a significant difference between the two cell treatments at fixed times. ***, ****; $P<0.001$ and 0.0001, respectively.
3.5 Non-viable cells increase following exposure to polymeric formula. Caco-2 cell viability was assessed by counting of trypan blue stained Caco-2 cells, with and without exposure to PF. Results are the mean ± SE of eight independent experiments. Raw data were analysed by 2 way repeated measures ANOVA. Asterisks indicate a significant difference between the two cell treatments at fixed times. ***, ****; \( P<0.001 \) and 0.0001, respectively.

3.6 Polymeric formula increases rates of apoptosis

The increased number of trypan blue-stained Caco-2 cells following exposure to PF indicated a higher rate of cell death. This could be due to accelerated differentiation resulting in apoptosis at of the cells at the villus tips. Conversely if the cells were dying by necrosis, this would indicate a toxic effect of PF. In order to investigate this, day 5 monolayers of Caco-2 cells treated with PF, or left untreated, were followed over time using live cell microscopy to track changes in the cell populations. Magic Red (Immunochemistry Technologies, USA) is a reagent that can move in and out of cells freely but becomes fluorescent in the presence of activated caspases, causing apoptotic but not necrotic cells to fluoresce red. Images were taken every five minutes over 10 hours, and a minimal volume of medium was added (400µL) to give a clearer image.

Untreated Caco-2 cells showed a more rapid initial increase in caspase activity than PF treated cells. But after 8 hours, cells 20% PF treatment showed higher caspase activity and more diffuse pattern of fluorescence (Figure 3.6). However, it was unclear whether the observed PF-related effect was real, or a result of increased light scattering through the PF. Accordingly, it remains to be determined if the increased rate of cell death observed in PF-treated Caco-2 cells was a result of increased apoptosis.
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Figure 3.6: Caspase activation in Caco-2 cells. Cell monolayers (untreated or treated with 20% (v/v) PF were stained with Magic red as a marker of apoptosis. Two hourly images are shown, and all images had the same length of exposure. These images are representative of two independent experiments.

3.7 Polymeric formula is associated with increasing IAP activity

The next step was to determine if the observation that the effect of 20% PF upon cell growth was associated with any significant change in the IAP activity over the same period of time. The results of these experiments (Figure 3.7) showed that IAP activity in the untreated cells did not increase over time. However, treatment of the cells with 20% PF caused a significant increase in IAP activity over time ($P<0.0001$). Consequently, IAP expression was significantly higher in PF-treated compared to untreated wells at 48 and 72 hours.

Figure 3.7: PF treatment increases cellular membrane associated IAP. Caco-2 cells were incubated with 20% (v/v) PF for up to 3 days, with IAP activity in the cell homogenates measured at 24 h intervals. Results are the mean ± SE of three independent experiments. Raw data were analysed by 2 way repeated measures ANOVA. Asterisks indicate a significant difference between the two cell treatments at fixed times. **, ***; $P<0.01$ and 0.001, respectively.
3.8 Polymeric formula increases membrane expression of IAP

Five day old Caco-2 cell monolayers incubated with 20% (v/v) PF for 48 and 72 hours were then lifted and the cells lysed in RIPA buffer. The proteins were separated by electrophoresis before being transferred to PVDF membrane and probed with monoclonal antibodies against IAP. Each blot was then reprobed with an antibody specific for GAPDH. UViBand software (UVITEC) was used to analyse each band volume by densitometry and thus the density of IAP staining was determined relative to the staining intensity of GAPDH in the same sample.

PF treatment was observed to increase cell-associated IAP staining at both time points (1.361 ± 0.487 and 1.901 ± 0.509 at 48 and 72 hours, respectively) when compared to untreated controls (0.710 ± 0.111). However, analysis of the raw data from three independent experiments did not detect a significant increase in IAP staining at either time point.

![Figure 3.8: PF causes an increase in IAP protein expression.](image)

Caco-2 cells grown on coverslips for 5 days were given fresh medium with increasing concentrations (0, 5, 10 and 20%) of PF for up to 72 hours. The cell monolayers were then fixed in 4% paraformaldehyde before being incubated with a monoclonal antibody against IAP. The cells were not permeabilised, enabling detection of IAP on the cell surface only. Binding of the primary antibody was subsequently detected using a FITC-conjugated secondary antibody, while the cell nuclei were stained with Hoechst. All images were taken using a Zeiss Z1 microscope at 20x magnification with a FITC
exposure time of 500ms. There was no detectable fluorescence on slides without the primary antibody at 500ms of exposure (not shown).

A marked difference in IAP protein expression between untreated and PF-treated cells was observed, as was a concentration-dependent effect of PF. In untreated Caco-2 cells, membrane-associated IAP expression was seen to be discrete and not evenly distributed. With each increase in PF concentration, the IAP staining became more widespread and diffuse. A similar effect was observed when fluorescence microscopy was used to monitor Magic Red staining of PF-treated cells. Collectively, these results suggest that PF may be responsible for increased scattering of light, but it was clear that higher PF concentrations caused an increase in IAP fluorescence, which is supported by previous results showing an increase in IAP activity.

![Image of fluorescence microscopy results](image)

**Figure 3.9. Polymeric formula increases membrane-associated IAP.** Caco-2 cells were (A) untreated, or treated with (B) 5%, (C) 10% of (D) 20% PF for 72 hours before being stained for membrane-associated IAP. Magnification 20x.
3.9 Polymeric formula causes a decrease in Caco-2 cell migration

Increased levels of PNP in treated Caco-2 cells supports the hypothesis that PF-treatment of gut epithelial cells may increase cell differentiation, contributing to the formation of a monolayer of cells that is maintained by cell-to-cell junctions [23]. To explore this idea further, Caco-2 cells were grown in a confined area for 24 hours, resulting in a circular monolayer of cells that was then grown for another 18 days with and without exposure to 20% (v/v) PF.

Previous studies have performed migration experiments using commercial kits or by cutting through the cells with crude instruments such as razor blades [51]. This approach was tried but was found to be ineffective as the Caco-2 cell monolayers were so tightly adherent to each other that cutting resulted in the cells lifting from the plates (data not shown). Accordingly, another method was developed to assess the effect of PF treatment on cell migration. Using the base of a pipette tip, a suspension of $1 \times 10^5$ cells were trapped within the circular area. After 24 hours the collar was lifted and the cells were incubated in medium with/without the addition of PF for 18 days (when untreated cells were noted to be approaching the edge of the well). The diameter of the monolayer was measured to the nearest half millimetre.

Results showed that exposure to 20% PF reduced cell migration by 44% (Figure 3.10). In the time it took untreated cells to spread an average of 5.9mm, PF treated cells had reached only 3.3mm ($P = 0.004$).

![Figure 3.10: PF decreases the rate of Caco-2 cell migration](image)

Figure 3.10: PF decreases the rate of Caco-2 cell migration. Caco-2 cells grown to confluence in a restricted space were slower to grow beyond this area when exposed to 20% PF for 18 days. Results are the mean ± SE of four independent experiments. **; $P < 0.01$ (by t-test).
3.10 Discussion

The first objective of this chapter was to measure the rate of Caco-2 cell proliferation and differentiation so that an appropriate time point could be chosen for further experiments. Cell numbers were seen to increase between days three and five, gradually transitioning from a proliferative to a slower rate of growth indicative of differentiation from day five onwards. Despite the observed changes in the rate of cell proliferation, cell death remained relatively constant as reported elsewhere [30, 33, 39]. Cell death varied between 5% and 10% for both concentrations when measured using trypan blue exclusion and flow cytometry. Cell viability did not appear to increase or decrease over time.

These results, which suggested that the cells were confluent but only partially differentiated informed the choice of using cells grown for five days in a 24 well plate cells as the time point to add PF. This is a similar time frame to that found in vivo as it reportedly takes between four to eight days for new enterocytes to move from the base of the crypt to the top of the villi [7]. Choosing partially differentiated cells at day five enabled relative increases and decreases in differentiation to be distinguished. These differences were to be measured at 24, 48 and 72 hour time points. Both seeding concentrations resulted in predictable rates of cell division and death, and a similar level of IAP activity. A concentration of $1 \times 10^5$ was chosen with an intervention time point of five days as this is when proliferation slows down, which suggests the cells are beginning to differentiate.

The next objective was to confirm that the cells were indeed beginning to differentiate as cell proliferation slowed. IAP levels, which are considered a marker of epithelial cell differentiation [28, 30], were quantified using paranitrophenol phosphate, a colourless substrate that becomes the yellow para-nitrophenol when acted upon by a phosphatase. Both membrane-associated and secreted IAP activity increased over time, indicating progressive differentiation, with no obvious difference relating to cell seeding concentrations. IAP activity in the cell supernatant was four times greater than membrane associated activity however activity in the supernatant was more difficult to detect due to the greater volume. This is consistent with an in vivo role for IAP as a secreted antimicrobial peptide, defending the epithelium against attack by adherent and/or invasive bacteria [29].
Polymeric formula was found to have a significant effect on Caco-2 cell proliferation and differentiation. Cell numbers decreased over time in the presence of PF, resulting in 50% less cells after 72 hours. This decrease in cell number was associated with an increase in cell death, which was three fold greater than that observed in untreated cells. Live fluorescence microscopy of caspase activity was used to explore the hypothesis that PF-treated cells were undergoing accelerated differentiation rather than toxin induced necrosis. After 10 hours of PF, there was a significant increase in caspase fluorescence consistent with an increased rate of differentiation. This is supported by a marked increase in IAP protein expression and enzyme activity in the PF-treated cells, given that IAP is widely considered a marker of epithelial cell differentiation [28, 30]. However, with both methods of fluorescence microscopy the signal was more diffuse in the PF-treated cells. This is likely to be due to increased light scattering from PF components that resist being washed off the cells.

PF was also shown to cause a 44% decrease in cell migration. This can be attributable to a decrease in cell proliferation and an increase in cell death as Caco-2 cells appeared to spread mainly through cell division. This is in constrast with results that have shown PF to support mucosal healing in vivo [9] and highlights one of the potential pitfalls of this in vitro reductionist model.

With the Caco-2 cell model established, and evidence to suggest that polymeric formula has a measureable effect on cell growth and differentiation, the next step was to investigate whether PF had a similar effect on a more biologically relevant model of the intestinal epithelium.
Chapter 4

The Caco-2 cell line is widely used to model the intestinal wall. Originally sourced from an adenocarcinoma of the colon [49], this cell line has been propagated in the laboratory for decades, with the cancer-causing mutations that the cells harbour retained and exacerbated by an in vitro environment that favours further anti-apoptotic and pro-survival change. Accordingly, using a cultured epithelial cell line such as Caco-2 cells may not accurately reflect how polymeric formula (PF) might act in vivo.

Intestinal epithelial cells originate from stem cells at the base of the crypts between villi. Enterocytes migrate over 4 to 8 days to the top of the villi where they are shed into the intestinal lumen [7]. The stem cells also produce goblet, enteroendocrine and Paneth cells which produce intestinal mucus, hormones and anti-microbial agents, respectively [26]. Each cell type is polarised, with different functions at the apical and basolateral surfaces. Collectively, the complexity of these cell types that comprise the intestinal epithelium challenges the generalisability of using monoclonal cell lines to study the gut.

In a favourable environment, the stem cell populations produce miniature intestinal epithelial structures that can easily used for controlled experimentation. These “enteroids” are an alternative cell culture model that more closely reflect the small intestine in situ as they contain many different cell types and are sourced directly from normal intestinal epithelium. The small intestine is removed from a mouse (or a biopsy collected from a patient during colonoscopy), with ion chelation and physical agitation used to remove crypts and villi from the submucosa. The villi are filtered out and the stem cell rich crypts are resuspended in an extracellular matrix (Matrigel) with the appropriate nutrients and growth factors.

This is important because in vivo these nutrients would be readily available through the local blood supply and/or directly from the intestinal lumen. For example, stem growth factors are derived from myofibroblasts beneath the basement membrane [46]. One of the most important of these is Wnt, which is present in low concentrations at the tips of villi and high concentrations in the crypts in order to prevent the stem cells from differentiating. The Wnt signalling cascade is enhanced by the addition of Noggin and R-spondin [47]. Epidermal growth factor (EGF) would normally be supplied to cells through the blood supply and the addition of this to the enteroid media has been
shown to increase stem cell proliferation, increasing the size of enteroids [47]. In addition, cells that are stripped from their extracellular matrix (ECM) are susceptible to apoptosis in order to prevent abnormal cell growth. To overcome this, enteroids are suspended in Matrigel. This contains collagen IV and laminin, thus mimicking the stroma surrounding intestinal crypts, and helps the cells that form the enteroids to survive outside their body of origin.

The objectives for this chapter were to:

1. Develop mouse enteroids as a more biologically relevant model of the small intestine
2. Investigate the effects of polymeric formula on murine enteroids

### 4.1 Culturing murine enteroids

Crypt cells isolated from the small intestine of a mouse were observed to close off into cysts by day 1 that continued to get larger as the stem cells continued dividing (Figure 4.1). By day 4 new crypts were seen projecting from the inner mass of cells, and at day 7, the most successful enteroids had multiple crypts extending from the central lumen At this point parts of the enteroid were sometimes obscured by loose clusters of cells generated within the enteroid.

It was observed that only some of the many crypts isolated from each mouse went onto to develop into large enteroid structures. Paneth cells within these structures were identified as darker cells at the base of the crypts, due to the high density of secretory granules they contain. The goblet cells were identified by their bulbous shape and their position intercalated between the enterocytes. It was also noted that a failure to consistently replenish the growth factors required for enteroid growth caused the structures to disintegrate into clumps of non-polarised cells (not shown).
Figure 4.1: Early and late mouse small intestine epithelial cell fractions. The first fractions are rich in villi (A) while later fractions are crypt rich (B) and contain the stem cell populations required for enteroid growth. Magnification 20x.

Figure 4.2: Development of enteroids over ten days. Mouse small intestine crypts were incubated in enteroid medium in Matrigel and images were taken every second day. Magnification 20x.
4.2 Enteroid response to PF

After 10 days in culture the growth rate of the enteroids was observed to slow with few new crypts being formed. At this point, fresh medium was added to the enteroids, with and without the addition of PF. In a matter of hours the crypt and lumen structure of the PF-treated enteroids became less defined before fully disintegrating, appearing more like aggregates of cells rather than enclosed monolayers with a visible lumen and crypts projecting outwards. The enteroids that were not exposed to PF did not show such changes (Figure 4.4).

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<tr>
<th>Time</th>
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<th>20% polymeric formula</th>
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Figure 4.4: Effect on polymeric formula (PF) on enteroid morphology. Ten day old enteroids were treated with 20% (v/v) PF for 6 hours. Images were taken at each hour interval and for each treatment condition, of which 4 representative images were chosen. Magnification 20x. This is representative of 2 independent experiments.

Images taken at a lower magnification (Figure 4.5) clearly demonstrate the global loss of cellular structures with PF treatment. As a result the cells more closely resembled
the loose clumps of cells surrounding enteroids. In contrast, the untreated enteroids retained their structure, with goblet and Paneth cells identifiable in the crypts. This loss of enteroid structure does not appear to be a beneficial effect of PF.

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**Figure 4.5: Changes to enteroid morphology after 6 hours of 20% PF treatment.** Gross changes in enteroid appearance with 20% (v/v) PF treatment could easily be identified (right) when compared to untreated enteroids (left). Magnification 4x.

Enteroids exposed to PF for 6 hours were fixed in paraformaldehyde and immunostained for evidence of IAP expression using a rabbit anti-IAP polyclonal antibody. Untreated enteroids incubated for the same period of time were also immunostained. Confocal microscopy revealed that the untreated enteroids contained IAP and that staining was more concentrated on the cell membrane (Figure 4.6).
It was difficult to find remaining enteroid-like structures among the cells following treatment with PF; any that were present were small and had a pattern of staining that was more similar to the isolated cells that exist around the enteroids. The length of exposure was not kept constant between slides so a comparison of IAP protein concentration could not be made, only observations of the distribution of the protein. Rather than the more the diffuse cellular staining of IAP in enteroids that hadn't been treated with PF, a more granular pattern of staining was observed in the PF-treated enteroids, with all cells apparently affected regardless of whether or not they retained
any enteroid features. The pattern of staining and cell behaviour appeared to mimic that observed within the loose clumps of cells around untreated enteroids.

![Image](image.jpg)

**Figure 4.7: IAP staining (yellow) of 10 day old enteroids treated with PF for 6 hours.** Images shown are representative of enteroids treated with PF for 6 hours before being fixed in 4% paraformaldehyde (PFA) and stained for IAP protein expression (yellow), with Hoescht used as a nuclear stain (blue). Image (A) shows a typical aggregate of cells following enteroid treatment with PF, whereas (B) shows the only cystic structure remaining. Scale bars 25µm.

One of the issues with this model is that the basolateral surface of the cells are exposed to the PF, whereas *in vivo* it is only the apical surface that would come in direct contact. Because of this, an attempt was made to overlay the newly isolated crypts on top of a collagen matrix instead of dispersing them within matrigel. With the same growth factors and nutrients present, the stem cells should have proliferated and produced a monolayer of intestinal epithelium, complete with multiple cell types and mucus production. However, the cells did not adapt readily to this environment and, accordingly, did not form monolayers on the collagen, even in the presence of conditioned media from isolated mouse myofibroblasts (Figure 4.8).
4.3 Discussion

The first objective of this chapter was to develop a method to culture enteroids as a model for studying the intestine. Crypts were isolated from murine small intestine as these were more readily available than human biopsies, and also produce a higher yield. The enteroids were sustained for up to three weeks but it was found that proliferation decreased after the first ten days. At this point the enteroids had reached 0.2 - 0.3mm in diameter and had multiple crypts extending out from the central lumen. The presence of Paneth cells and goblet cells could be seen by live microscopy. These and other cell types have been identified in enteroids by the presence of villin on enterocytes, Mucin-2 within Goblet cells, lysozyme release by Paneth cells and Chromogranin A by enteroendocrine cells [26].

Ten days was chosen as the timepoint for the second objective, to investigate the effect that 20% (v/v) PF has on murine enteroids. The intention was to replicate the experiments undertaken using Caco-2 cells and measure the cellular response over 72 hours. However, the 10 day old enteroid structures disintegrated within six hours of PF being present in the cell medium. The treated enteroids were stained for IAP, and although relative amounts of expression could not be determined, patterns of expression had drastically changed. Furthermore, almost all the crypts and enterocyte walls had dissociated. The change was relatively rapid compared to the effect the same concentration of PF had on Caco-2 cells, which took between one and two days to be markedly different from untreated cells.
The mechanism underlying the rapid disintegration of PF-treated enteroids remains to be investigated but it appeared to mimic what happened when the growth factors essential for enteroid growth and survival were depleted (not shown). This leads to the hypothesis that the PF may be binding to and/or competing with one of the growth factors that are essential for enteroid growth. If so, PF is likely to be interfering with the Wnt signalling pathway, which drives stem cell proliferation and movement of transit amplifying cells up the crypt. As the enterocytes move up the villus, Wnt concentrations decrease and bone morphogenic protein (BMP) concentrations increase, the cells stop proliferating and instead begin to differentiate [52]. PF has been shown to delay degradation of IκBα, which prevents NF-κB from migrating to the cell nucleus, causing anti-inflammatory effects [43]. Increased NF-κB activity has been associated with increased Wnt signalling in mouse enteroids [53]. Therefore reduced NF-κB activity due to PF treatment may inhibit this crucial pro-proliferation and de-differentiation pathway. This most likely explains the observed pro-differentiation effects in both Caco-2 cells and primary enterocytes. However, it is apparent that cultured enteroids are considerably less robust than transformed cells, again supporting the hypothesis that components of PF may perturb the enhanced growth requirements of the enteroids.

An alternative method that involved the culture of a primary enterocyte monolayer on collagen was investigated as a means to explore the effect of PF treatment [46, 54]. This method has many of the advantages of closed enteroids such as a self-regenerating population of multiple non-transformed cell types but it also enables stimuli to be easily added to the apical or luminal surface and was considered to potentially provide a better model for imitating dietary interventions such as PF. However, the “flat” enteroids did not form the complex secondary structures such as crypts and tight monolayers seen in the matrigel enteroids under similar conditions. There was not sufficient time to determine the cause.

The failure of the Matrigel enteroid model to evaluate the effects of PF is currently under investigation. The first goal will be to determine a dose response relationship of enteroids to PF, with the ultimate goal being repeating these experiments with enteroids derived from human biopsies.
Chapter 5. Results

Polymeric formula has a demonstrable effect on cell differentiation (Chapter 3) but the components responsible for this effect are currently not known. One promising candidate is calcitriol or active vitamin D, which is listed as an ingredient of Osmolite 1 Cal (Abbott Nutrition), the polymeric formula used for this research.

Vitamin D deficiency is commonly seen in IBD [51, 55-56], and VDR knockout mice have a significant reduction in the ATG16L1 protein, one of the main risk genes for CD [56]. Most individuals get almost their entire required daily intake through a reaction between UV and vitamin D precursors in the skin [32]. However, enteral nutrition formulas have to provide the recommended amounts of vitamins and minerals under the assumption that there is no other source. Therefore a relatively high amount of active vitamin D is consumed by patients taking enteral nutrition exclusively.

Almost all known actions of vitamin D are mediated through the VDR [33, 56]. Caco-2 cells, like normal enterocytes, have a functional VDR at all stages of differentiation [30]. Therefore if PF is acting through the VDR in normal cells, then it is likely that the effects on Caco-2 cells would be mediated through the same pathway. Like PF, vitamin D is known to inhibit activity of NF-κB [57], which has been shown to affect the Wnt pathway [32, 53]. Wnt and downstream signalling is important for regulation of cellular proliferation and differentiation [53] and therefore the vitamin D present in PF could potentially be mediating its pro-differentiation effects.

Butyrate also reportedly signals through the VDR [33], resulting in a dose-dependent increase in IAP activity, CEA protein concentration and trans-epithelial electrical resistance (TEER) [39]. Butyrate also appears to drive crypt-to-villous differentiation in these cells [39].

It was hypothesised that the observed PF-related effects on cell differentiation were being mediated through the VDR. To investigate this, a vitamin D receptor inhibitor (VDRI) was obtained from the Teijin Institute in Japan. This VDRI (TEI-9647) has been shown to antagonise the VDR in human osteoclasts and monocytes [58-60]. However, there is no evidence to date regarding the ability of TEI-9647 to inhibit the VDR in Caco-2 cells. Butyrate and vitamin D were used as controls for these experiments.
Both are shown mediate an increase in Caco-2 differentiation through the VDR [33] and to increase its expression [56] with butyrate shown to be more potent [33].

The objectives for this chapter were to:

1. Measure IAP activity in Caco-2 cells exposed to vitamin D or butyrate.
2. Evaluate the effectiveness of a vitamin D receptor inhibitor to reduce IAP activity in vitamin D and/or butyrate-treated Caco-2 cells, and to determine the extent to which the effect of PF is mediated through the vitamin D receptor.

5.1 Butyrate causes a marked increase in IAP activity.

IAP levels were measured in butyrate and vitamin D-exposed Caco-2 cells as a first step to determining their potential as controls to confirm the effectiveness of the VDR inhibitor in this cell line. Increasing concentrations of butyrate and Vitamin D were added to 5 day old Caco-2 cell monolayers for 72 hours, with IAP activity measured as a marker of cellular response. Vitamin D was added at concentrations of 20nM, 40nM and 80nM, which are similar to the concentration present in 20% (v/v) PF, and also within the range of concentrations reportedly used in other studies to activate the vitamin D receptor in cultured cell lines [51].

Butyrate is known to cause an increase in the rate of differentiation at concentrations of 1mM to 2mM [38, 39]. This effect increases with dose but beyond 4mM butyrate becomes toxic to the cells and rates of cell death increase accordingly [38, 39]. Luminal butyrate produced by intestinal bacteria can reach 20mM but the protective mucus layer also reduces concentrations that reach the epithelium [39]. Butyrate was tested at 2mM, 4mM and 8mM, in order to observe the range of effects that butyrate is known to have.

Vitamin D and butyrate increased IAP activity at all concentrations tested (Figure 5.1). Vitamin D had the least effect, and it was not significantly different from that measured in untreated controls. In contrast, butyrate had a significant dose-dependent effect on IAP activity ($P<0.05$). Furthermore, the highest concentration of butyrate tested (8mM) induced the cell to produce 3.4µM of PNP per million cells, which was significantly higher than the 1.3µM PNP per million cells produced in response to 20% (v/v) PF ($P<0.05$).
5.2 High concentrations of butyrate causes Caco-2 cell death

These results showed butyrate to be a more potent stimulator of IAP activity than vitamin D and therefore a more suitable control for evaluating the effectiveness of the VDRI in Caco-2 cells. However, there is evidence to suggest that 8mM butyrate may be cytotoxic [38, 39], which meant it was important to determine the optimal concentration to induce IAP expression with minimal cell death.

Five day old Caco-2 cell monolayers were incubated with to 0mM, 2mM and 8mM of butyrate. Live microscopy was used to observe visual signs of cell apoptosis and death that included membrane blebbing, cell rounding and/or lifting [61]. The microscope was set to take images every 5 minutes using differential interference contrast (DIC) microscopy. As shown in Figure 5.2, the untreated and 2mM butyrate treated cell monolayers did not show any noticeable change in cell morphology over 25 hours. In contrast, exposure to 8mM sodium butyrate had a noticeable effect on the cells, with many lifting off the plate over the course of the experiment.
Figure 5.2: Dose-dependent effect of butyrate on Caco-2 cell monolayers. Five day old Caco-2 monolayers were incubated in the presence of 0mM, 2mM and 8mM butyrate and differential interference contrast (DIC) images were taken over 25 hours. Images shown were taken at 5 hour intervals and are representative of one experiment.

Despite inducing the highest levels of IAP activity, microscopy suggested that 8mM butyrate was causing the cell monolayers to disintegrate. Accordingly, five day
monolayers of Caco-2 cells were incubated with all three concentrations of butyrate. Cell numbers and viability were determined by counting cell numbers and the percentage of trypan blue-stained cells, respectively. Following exposure to 4mM butyrate, the number of Caco-2 cells per well was found to be similar to that of cells exposed to 20% (v/v) PF for the same period of time (Figure 5.4). Likewise, the percentage of dead cells was not higher in 4mM butyrate treated cells when compared to cell death rates in PF-treated cells (Figure 5.5). Accordingly, a concentration of 4mM butyrate was chosen as a control for the effectiveness of TEI-9647 in blocking the VDR.

While butyrate was shown to be a more potent stimulator of IAP activity, vitamin D is still the physiological ligand to the vitamin D receptor and research by others suggests that it should have had a greater magnitude of effect [33]. For this reason, cell responses in the presence of 80nM vitamin D, the highest concentration tested, were also measured in the following experiments.

### 5.3 TEI-9647 does not block PF-induced increase in IAP activity

A 4mM concentration of butyrate and 80nM Vitamin D were used to stimulate IAP release by five day old monolayers of Caco-2 cells, with and without the addition of the VDR inhibitor TEI-9647. In addition, the ability of TEI-9647 to block IAP release by Caco-2 cells in response to 20% (v/v) PF was assessed. The TEI-9647 inhibitor was provided at a concentration of 1mM dissolved in ethanol with a recommended dilution of 1:1000 for cell culture experiments. To optimise the response and observe a dose response, TEI-9647 was used in these experiments at half (0.5 µM) and at double (2µM) this concentration. Ethanol diluted 1/1000 reportedly has no significant effect on Caco-2 [33] but because some cells were incubated with VDRI at 2µM concentration, controls included cells exposed to a 1/500 dilution of ethanol in cell culture media, using the same experimental parameters. Cell numbers and viability in these ethanol controls did not differ from untreated cells (not shown).

Treatment of Caco-2 cells with TEI-9647 was associated with a dose-dependent decrease in IAP activity in butyrate and vitamin D-stimulated cells (P<0.05), with this effect was most notable in the presence of butyrate (Figure 5.3). In contrast, the VDRI had no effect on IAP levels in Caco-2 cells following exposure to 20% (v/v) PF.
5.4 TEI-9647 does not affect PF induced decrease in cell number

The VDRI was capable of reducing IAP activity under all treatment conditions except PF. Butyrate and PF also cause changes in Caco-2 proliferation and viability which may involve cellular pathways also influenced by VDR. Any effect of the VDRI would be expected to reduce a PF or butyrate-induced decline in cell number and increase in cell death.

80nM vitamin D caused a non-significant increase in cell number from around 0.34 million cells per well to around 0.42 million cells. 4mM butyrate and 20% PF both caused cell number to drop to around 0.2 million cells, as expected from earlier experiments (Figure 5.1). TEI-9647 had no effect on PF, vitamin D or control cell number however a 50% reduction in cell number was observed in butyrate-treated cells incubated with 2µM VDRI (P<0.01). The significant effect of the VDRI on 4mM butyrate, without any change in response by PF treated cells, indicates that the VDR does not mediate PF-induced changes in cell proliferation.
Vitamin D (80nm), 4mM butyrate and 20% (v/v) polymeric formula were added to 5 day old Caco-2 monolayers for 72 hours without and without the addition of VDRI at 0.5µM and 2µM, and cell numbers were counted by haemocytometer. Results are ± SEM of 3 independent experiments. Raw data for each treatment group were analysed separately by repeated measure ANOVA, and asterisks linked by bars indicate significant differences between the different concentrations of inhibitor within each group. **, P<0.01.

5.5 TEI-9647 has no effect on Caco-2 cell viability

80nM Vitamin D treatment did not appear to affect cell viability, ranging between 5% and 10% as seen with untreated cells. 4mM butyrate caused an increase in cell death to 16%, while treatment with 20% PF resulted in approximately 21% of the cells dying. This PF-mediated increase in cell death was significant when compared to untreated controls (P<0.01; Figure 5.5).

Addition of the VDRI had no apparent effect on the rate of cell death in untreated cells or cell treated with either 20% PF or 80nM vitamin D. However, increasing concentrations of TEI-9647 added to 4mM butyrate treated cells resulted in a notable decrease in cell death, and this is consistent with butyrate-induced cell death being mediated through the VDR (Figure 5.5).
Figure 5.5: VDRI TEI-9647 does not affect Caco-2 cell viability in the presence of stimuli. Vitamin D (80nM), 4mM butyrate and 20% (v/v) polymeric formula were added to 5 day old Caco-2 monolayers for 72 hours without and without the addition of the VDRI at 0.5µM and 2µM, and cell viability was determined by trypan blue exclusion. Results are ± SEM of 3 independent experiments. Raw data for each treatment group were analysed separately by repeated measure ANOVA. There were no significant differences between the different concentrations of inhibitor within each group.

5.6 Discussion

The first objective of these experiments was to determine suitable concentrations of vitamin D and butyrate to be used as positive controls for assessing the ability of TEI-9647 to inhibit the VDR in Caco-2 cells. This was done by measuring increase in IAP activity after 72 hours and comparing the results with levels expressed by cells treated with 20% (v/v) PF.

Both vitamin D and butyrate caused a dose-dependent increase in IAP activity. This increase was demonstrably clear with butyrate but, in marked contrast, the magnitude of the effect in cells stimulated with vitamin D was very small. This was unexpected as the concentrations of vitamin D used were within the range reported elsewhere in experiments using Caco-2 cells [51]. Additionally, the concentrations of vitamin D were similar to those which would be expected in cell medium containing 20% PF. This marked difference may have been due to a loss of biological activity in the vitamin D, either by oxidation, or through exposure to ultraviolet light. However, another possibility also exists. In some studies vitamin D has been found to have no or little effect on its own in affecting markers of epithelial function such as tight junctions or AMP expression, but capable of attenuating the effect of an inflammatory stimulus [20,
Thus, the absence of NF-κB stimulation may have limited the stimulatory effect of vitamin D.

The greatest increase in IAP activity was in cells treated with 8mM butyrate. However, subsequent experiments showed that this concentration of butyrate has a cytotoxic effect on Caco-2 cells. While untreated and 2mM treated cells showed little difference over 25 hours, DIC microscopy of 8mM butyrate showed a clear difference in cell morphology, with a loss of cells from the monolayer, rounding and blebbing of cell membranes. This was confirmed by cell number and viability counts. IAP activity was also substantially increased in Caco-2 cells treated with 4mM butyrate. Similar counts of 4mM butyrate-treated cells revealed that this concentration was no more toxic to Caco-2 cells that 20% (v/v) PF and this concentration was subsequently chosen for ongoing experiments.

The second objective was to confirm that TEI-9647 is an effective VDRI in Caco-2 cells, and to ascertain the contribution of the VDR to the observed PF induced pro-differentiation response in these cells. The addition of TEI-9647 was shown to cause a significant decrease in IAP activity at 72 hours in the presence of either positive control (butyrate or vitamin D), and when added to untreated cells. This was most notable when the higher concentration of TEI-9647 was added to cells stimulated with 4mM butyrate, with IAP levels falling by approximately half. In marked contrast, the effect of 20% PF on IAP levels was not affected at either VDRI concentration, which suggests that the VDR does not mediate the PF induced effect in Caco-2 cells. Ideally, this hypothesis would have been explored further by using increased concentrations of the VDRI. However, TEI-9647 was provided by the company as a solution in absolute ethanol, and the likelihood of an ethanol-mediated effect on the cells ruled out this approach.

As well as IAP activity, cell proliferation had also been shown to change in the presence of PF. Cell proliferation increased with 80nM vitamin D but decreased with 4mM butyrate, similar to that seen in Caco-2 cells treated with 20% PF. Treatment with TEI-9647 blocked the butyrate-mediated effect on cell growth, and this effect was observed to be concentration-dependent. In contrast, VDRI-treatment of Caco-2 cells did not override the inhibitory effect of 20% PF on cell proliferation. Also, the VDRI did not cause a significant change in rates of cell death.
This lack of response of PF-treated Caco-2 cells to VDR inhibition may provide an explanation as to why 10nM vitamin D is shown to cause an increase in Caco-2 cell migration [51] whereas treatment with 20% PF as the stimuli showed the opposite effect (Chapter 3). Likewise, Vitamin D treatment was shown to cause opposing changes to cell proliferation, an important factor in the rate of Caco-2 cell spread. Collectively, these results suggest that it is unlikely that the observed effect of PF on Caco-2 differentiation is mediated through the VDR.
Chapter 6. Discussion

6.1 Summary of results

As shown in other studies, Caco-2 cells were found to spontaneously differentiate and display many of the characteristics of enterocytes that included the formation of an adherent monolayer and expression of IAP [30, 39]. With two different seeding concentrations, 5 x 10^4 and 1 x 10^5 cells per 2cm^2 well, the cells initially underwent a phase of rapid proliferation. After 5 to 6 days an increase in cell density and contact inhibition prompted the cells to differentiate. The concentration of non-viable cells varied between 5% and 10%, and did not increase with time.

Measurement of IAP activity was used as a known marker of Caco-2 differentiation [28, 30]. Levels in both membrane and secreted fractions increased as cell numbers increased, and continued to increase as cellular proliferation slowed with the start of differentiation. Total IAP activity was much higher in the supernatant when compared to the corresponding cell lysates, which is consistent with directed IAP secretion. However due to the extensive dilution in the cell medium, changes in secreted IAP activity were more difficult to detect. This was further compounded when investigating the effect of the highly opaque polymeric formula (PF).

Seeding each well with 1 x 10^5 cells and incubating for a period of five days was chosen as the time-point at which to add stimuli. At this point the cells had formed a confluent monolayer but were only partially differentiated so could still respond to pro-differentiation stimuli. Treatment with 20% (v/v) polymeric formula (PF) over 72 hours resulted in a 50% decrease in cell number. PF was also shown to cause a 44% inhibition of cell migration over 18 days, which can be attributable to the decrease in Caco-2 cell proliferation. By 72 hours there was also a threefold increase in cell death, which was shown to be related to increased apoptosis over the first 10 hours rather than toxic necrosis. This increase in apoptosis is consistent with an increased rate of differentiation as apoptosis and exfoliation from the tip of the villus is the end stage in enterocyte differentiation, to make way for newly differentiated cells. Acceleration of this process was supported by the increase in IAP activity and protein expression with PF treatment. This was statistically significant by 48 hours and showed a further increase at 72 hours.
An additional model of small intestine epithelium was developed, the enteroid. Stem cell rich crypts were harvested from mice, as these are both easier to obtain and have higher rates of success than human biopsy enteroids [47]. The mouse crypts were incubated within an extracellular matrix; nutrients and growth factors were added to the culture medium to stimulate stem cell proliferation and survival. These crypts were found to close over and become cysts over the first day, then to increase in size to between 0.2 and 0.3 mm and generate new crypts from a central lumen. Enteroids are more similar to normal intestinal epithelium than Caco-2 cells as they contain the full range of cell types that produce the enzymes, mucus and anti-microbial peptides present in a functioning intestine [26]. In these enteroids, mucus secreting goblet cells could be identified by their characteristic shape, and AMP producing Paneth cells could be identified by their location at the base of crypts and due to their darker appearance under bright-field microscopy. However between 10 days and 3 weeks the enteroids no longer increased in size or complexity despite replacement of growth factors every 2 days. For this reason, day 10 was chosen as an appropriate time point for investigation of the effect of 20% (v/v) PF in the enteroid model.

Within hours of PF treatment, the enteroid structures disintegrated into loose aggregates of cells. This occurred much more rapidly than the effects of PF on Caco-2 cells, which occurred over two to three days. Changes in the pattern of IAP protein expression were observed using confocal microscopy, where 6 hours of 20% PF treatment resulted in a granular pattern of staining while untreated enteroids had a more uniform signal. Almost all layers of the PF-treated enterocytes dissociated into clusters of non-polarised cells, which was similar to what was observed when enteroids were briefly deprived of necessary growth factors. The lead to the hypothesis that reduced activity of the Wnt signalling pathway may be involved. PF causes a delay in degradation of IκBα, which binds to NF-κB and prevents it from translocating to the cell nucleus where it causes up-regulation of pro-inflammatory gene expression (de Jong). The Wnt cellular pathway has been shown to be promoted by an increase in NF-κB activity [53], thus the reduced proliferation and pro-differentiation effects of PF could be mediated by a reduction in NF-κB and Wnt downstream effectors.

Growing mouse crypts on a collagen layer rather than within a matrix was attempted in order to develop a two dimensional primary cell monolayer model. Collagen grown enteroids are similar to those immersed in Matrigel in that they reportedly contain multiple types of self-regenerating and differentiating cells and are therefore representative of normal intestinal epithelium [46, 54]. It was speculated that this
model might even be potentially more relevant when experimenting with dietary treatments with the ability to add stimuli to the luminal surface, as would occur in vivo. However the stem cells did not proliferate and form monolayers over the collagen surface despite having the enteroid growth factors present in the cell medium. This remains to be investigated.

Vitamin D was considered as a contributor to the mechanism of PF as it has been shown to have similar effects. These include inhibition of IκBα degradation, down-regulation of inflammatory cytokine production [20, 23, 43] and an increase in IAP activity [33]. Vitamin D deficiency is commonly seen in CD, of which PF is a treatment [20, 51, 55, 56]. The VDR mediates most known functions of vitamin D [56], and a VDR antagonist was obtained to determine inhibition of the VDR would likewise inhibit the effects of PF.

Vitamin D and butyrate were used as a positive controls for determining the effectiveness of VDR inhibition with TEI-9647. Both are known to promote enterocyte differentiation through the VDR [33, 39, 56]. A dose response effect on IAP activity after 72 hours was used to determine the optimum concentrations. The highest concentration of butyrate, 8mM, resulted in the greatest increase in IAP activity, however this occurred at the expense of cell viability, which was shown to be markedly affected within 25 hours of exposure. 4mM of butyrate stimulated a large increase in IAP activity but without the corresponding increase in cell death.

TEI-9647 did not affect cell viability in the presence of any of the cell treatments. Yet it halved the effect of 4mM butyrate on cell proliferation and IAP activity. This effect was significant and displayed a dose response. TEI-9647 also caused a significant decrease in IAP activity in both 80nM vitamin D treated and untreated cells. In contrast, the presence of TEI-9647 had no effect on PF-mediated cell proliferation and/or IAP activity, indicating that the accelerated differentiation of Caco-2 cells in the presence of PF is not primarily mediated through the vitamin D receptor.

6.2 Future directions
In order to further understand the interactions between PF and enterocyte differentiation, these experiments need to be retested with a more complete model that would include knocking out the VDR through the use of RNA interference, gene knockout or use of a stronger antagonist. Additional inhibition of NF-κB and/or Wnt
signalling would support or dispel the hypothesis that reduced activation of this cellular cascade is mediating the effects seen in both Caco-2 and enteroids.

Further development of the enteroid model is also necessary, with a greater understanding of the cause(s) of its limitations required to enable more realistic interactions to occur. This work is underway, starting with a project aimed at measuring the dose response of enteroids to multiple concentrations and fractions of PF.
Bibliography


antimicrobial protein 18 expression by human colon epithelium. Infection and immunity 70 (2):953-963


Vitamin D(3) promotes the differentiation of colon carcinoma cells by the induction of E-cadherin and the inhibition of beta-catenin signaling. The Journal of cell biology 154 (2):369-387


43. de Jong NS, Leach ST, Day AS (2007) Polymeric formula has direct anti-inflammatory effects on enterocytes in an in vitro model of intestinal inflammation. Digestive diseases and sciences 52 (9):2029-2036

44. Keenan JI, Hooper EM, Tyrer PC, Day AS (2013) Influences of enteral nutrition upon CEACAM6 expression by intestinal epithelial cells. Innate immunity


