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MICROBIALLY-REGULATED INTESTINAL EPITHELIAL HMGB1: STRESS, CELLULAR ENERGY PRODUCTION AND SURVIVAL

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DEDICATION

I dedicate this to

Jeannette Messer, Ph.D.,

and Candace Cham, Ph.D.,

without whose tireless effort and

constant attention to me and my work,

this thesis would not have been completed.

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List of Abbreviations

3-D: three dimensional

ACOG: American College of Gastroenterology

ADF: Advanced Dulbecco's Modified Eagle's Medium Nutrient Mixture F12

Akt: also known as Protein kinase B

AMP: Antimicrobial Peptides

ANOVA: Analysis of Variance

AP: activator protein

Atg5: Autophagy protein 5

ATG16L1: Autophagy 16 Like-1

ATP: Adenosine triphosphate

B.: Bifidobacteria

B. subtilis: Bacillus subtilis

CARD15

CD: Crohn's Disease

CDI: *Clostridium difficile* infection

CpG: nucleotide sequence of cytosine followed by guanine

CDEIS: Crohn's Disease Endoscopic Index of Severity

Colonoids: three dimensional structures grown from stem cells isolated from the colon

DCA: deoxycholic acid

DNA: Deoxyribonucleic acid

DPBS: Dulbecco's Phosphate-Buffered Saline

DSS: Dextran Sodium Sulfate

E. Coli: Escherichia Coli

EDTA: Ethylenediaminetetraacetic Acid

EGF: Epidermal Growth Factor

ELISA: Enzyme Linked ImmunoSorbent Assay

EMA: European Medicines Agency

Enteroids: three dimensional structures grown from stem cells derived from the small intestine

f/f or fl/fl: floxed

f/f vil-cre or fl/fl vil-cre: floxed villin-cre

FDA: Food and Drug Administration (United States Agency)

FLA: flagellin

FMT: fecal microbial transplantation

FODMAP: fermentable oligosaccharide, disaccharide, monosaccharide and polyol

FOS: Fructooligosaccharides

GAPDH: Glyceraldehyde 3-phosphate dehydrogenase

GF: germ-free or gnotobiotic

GSK3β: Glycogen synthase kinase 3 beta

GWAS: genome wide association studies

HEPES: 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

HMGB1: High Mobility Group Box 1

H. pylori: *Helicobacter pylori*

HTS: High-Throughput Screen

IACUC: Institutional Animal Care and Use Committee

IBD: Inflammatory Bowel Disease

IEC: Intestinal Epithelial Cells

IL: Interleukin

L: Lactobacilli

LGG: *Lactobacillus rhamnosus GG*

LPS: Lipopolysaccharide

LTA: Lipoteichoic Acid

MDP: Muramyl DiPeptide

mRNA: messenger ribonucleic acid

MyD88: myeloid differentiation primary response protein

N.A.: North America

NF-kB: nuclear factor kappa-light-chain-enhancer of activated B cells

NCT: National Clinical Trial

NMDA: N-methyl-D-aspartate receptor

NOD: Nucleotide- binding oligomerization domain-containing protein

ODN: oligodeoxynucleotides

Organoids: generic term for three dimensional structures grown from stems cells derived from an organ

p62/SQSTM-1: ubiquitin binding protein p62 also known as sequestosome-1

PI3K: Phosphoinositide 3 Kinase

PMSF: Phenylmethylsulfonyl fluoride

PTEN: phosphatase and tensin homolog

Organoids: generic term for three dimensional structures grown from stems cells derived from an organ

Rep: Replicate

S. aureus: Staphylococcus aureus

S. typhimurium: Salmonella typhimurium

SPF: Specific pathogen-free

TLR: toll-like receptors

TNF: Tumor Necrosis Factor

TNBS: trinitrobenzenesulfonic acid

UC: Ulcerative Colitis

VSL#3: probiotic formulation of *Streptococcus thermophiles*, *Bifidobacterium breve*, *Bifidobacterium longum (recently reclassified as B. lactis according to vsl3.com)*, *Bifidobacterium infantis (recently reclassified as B. lactis according to vsl3.com, Lactobacillus acidophilus*, *Lactobacillus plantarum*, *Lactobacillus paracasei*, *Lactobacillus delbrueckii subsp. bulgaricus*

(recently reclassified as L. helveticus according to vsl3.com)

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Abstract

Inflammatory bowel diseases (IBD) are diseases of intestinal epithelial cell (IEC) death and mucosal damage. High mobility group box 1 (HMGB1) is a multifunctional protein whose intracellular expression is decreased in IEC from active IBD lesions. During microbial stress, intracellular HMGB1 regulates the cellular autophagy/apoptosis checkpoint to promote cell survival. Thus, HMGB1-regulated cell survival pathways represent potential therapeutic targets for this disease.

In this study, treatment of primary IEC (enteroids) with the bacterial components muramyl dipeptide (MDP), lipopolysaccharide (LPS), flagellin (FLA), lipoteichoic acid (LTA) or the bacteria DNA CpG-ODN increased HMGB1 protein levels. However, these same microbial stimuli activated apoptosis in HMGB1-deficient cells. Treatment with MDP likewise increased ATP production and Akt phosphorylation in wild type, but not HMGB1-deficient cells. We utilized this model of HMGB1-deficient enteroids to screen the Prestwick Chemical Library for drugs with the capacity to increase ATP production during MDP-induced cellular stress. The goal was to identify drugs that could increase survival of HMGB1-deficient IEC during microbial stress, a model of the mucosal epithelium of patients with active IBD. Nineteen drugs satisfied screening criteria for increased ATP production. One candidate drug, lovastatin was investigated further and found to restore Akt phosphorylation and autophagy in MDP-stressed HMGB1-deficient cells. Therefore, we developed a novel drug screening and validation pipeline to rapidly identify existing drugs or novel compounds that potentially preserve IEC functions and survival during IBD. This pipeline and the drugs identified through the screening could

provide new opportunities for IBD therapies that improve patient outcomes while minimizing side effects related to immunosuppression.

Chapter 1: Introduction

Intestinal Structure and Function

The intestine is a tubular, mucinous and muscular organ, part of the gastrointestinal tract (which includes the stomach as well as the intestine), performing the major activities of absorbing nutrients and protecting the organism from the environment. The small intestine, comprised of the duodenum, jejunum and ileum, in that order descending from the stomach pylorus, performs the majority of the absorptive functions on the chyme (the partially digested matter from the stomach). Thus disease in this area may inhibit proper nutrition for the organism, leading to nutrient deficiencies or malnutrition. The large intestine, connected from the small intestine through the ileocecal valve, concentrates the luminal contents (food and environmental components inside the tube) into the waste stream for excretion from the organism. Dehydration of the fecal matter and storage of the waste prior to elimination occurs in this distal and final end of the gastrointestinal tract (Kibble and Halsey 2015).

The functions of protection and digestion and absorption in the intestine require the four layers of the intestinal wall (serosa, muscularis externa, submucosa and mucosa). The serosa provides continuity with the peritoneal mesentery. Both the serosa and submucosa are connective tissue. The serosa is the entry point for blood vessels, external nerves, and exocrine gland ducts pass to allow major conduits of nervous, immunological and cardiovascular communication between the body and the intestine. Above the serosa, lies the muscularis externa, which utilizes two major smooth muscle

cell layers to perform peristalsis, the actions which propel the luminal contents (mainly food) down the intestinal tract. The next layer is the submucosa, which houses vasculature and lymphatic ducts for the nutrient absorptive and waste elimination properties of the gut. Finally, the layer that comes into contact with the environment (the luminal contents, the food, bacteria, and other non-food stuffs that entered the mouth of the organism) is the epithelium. These intestinal epithelial cells (IEC) form a single layer lining the invaginated morphology. This folded structure increases the surface area of the intestine that comes into contact with the environment. Endocrine cells within the epithelial layer excrete hormones that affect the function of the intestinal tract. The folds which form tubular structures comprise the exocrine glands, which secrete mucus, electrolytes, water and digestive enzymes (Kibble and Halsey 2015). The IEC thus directly interact with the internal environment, especially bacteria, of the intestine as well as signal to other parts of the intestine and finally to other organs.

Intestine and Microbiota

The intestinal epithelial cells lining the intestine come into contact not only with the food to digest and absorb nutrition, but also a vast ecosystem of microorganisms, bacteria, fungi, viruses, and potentially other environmental contaminants or non-digestible debris. The microorganisms, or microbiota, play a major role in the health of the organism or host, which needs to tolerate the massive community of residents or eliminate pathogenic ones. Through the interaction between microbes and the neonatal host, the microbiota educates the immune system and contributes to its maturation as

the host's intestine develops from birth (Sommer and Bäckhed 2013). Beneficial microbes prevent pathogens from colonizing as well, directly preventing infection (Kamada et al. 2013). Microbes adhere to the mucus layer via lectins and glycosidases and stimulate the production of antimicrobial peptides (AMPs) (Sommer and Bäckhed 2013). The microbiome has also been associated with bone density, adipose tissue, organ development and morphogenesis, and behavior as well as metabolism through various animal studies (Sommer and Bäckhed 2013). Because the microorganisms have an extensive metabolome, various metabolites are under study as to the mechanisms of these connections. For example, microbes make vitamins and amino acids, biotransform bile, break down many carbohydrates that the host does not (such as inulin, cellulose, pectins, gums and others), and form short chain fatty acids which provide nutrition to the host's cells, while other metabolites like gases and secondary bile acids typically are associated with apoptosis or aberrant cell proliferation (Sommer and Bäckhed 2013; Vipperla and O'Keefe 2012). Various receptors sense the microorganisms and their components and metabolites and provide the means for crosstalk between microbe and host. Though the explicit connections remain unclear, studies in colorectal cancer and inflammatory bowel disease show that alterations in the bacterial community impact intestinal disease (Vipperla and O'Keefe 2012; Ullman and Itzkowitz 2011). Even diseases that one does not necessarily typically associate with the intestine appear to be related to the microbiome of the gut: these diseases include asthma, arthritis, and cardiovascular disease as well as obesity (Sommer and Bäckhed 2013). The relationship between the intestinal epithelium and the bacteria define the

health of the host organism by determining how well the organism lives within its environment and is able to obtain the proper nutrition to sustain healthy functioning.

Inflammatory Bowel Disease

Characteristics of Inflammatory Bowel Diseases

While intestinal bacteria affect the health of the intestine, they also impact the development of intestinal disease, including inflammatory bowel diseases (IBD). Inflammatory bowel diseases are multicharacteristic in nature. The typical symptoms include diarrhea, rectal bleeding, abdominal pain, weight loss or intestinal obstruction associated with swollen and damaged intestinal mucosa (Neurath and Travis 2012). The two phenotypes, Ulcerative Colitis (UC) and Crohn's Disease (CD), differ based on inflammatory pattern and localization observed in the reddened mucosa (N.A. Society for Pediatric Gastroenterology 2007). Samuel Wilks (Wilks 1859) first compared the pathology of UC to that of mercury poisoning. The histological definition of UC involves ulceration and damage to the mucosa in the colon and rectum area. Crohn's disease, however, originally defined as a terminal ileum disease in 1932 (Crohn 1932) usually describes inflamed small intestine, but may spread to other gastrointestinal areas as well. Its transmural nature of lesions, abscesses, fistulas and strictures, and occasional intestinal obstructions typically do not occur in UC (N.A. Society for Pediatric Gastroenterology 2007).

Epidemiology

Over 1.2 million Americans and 2.2 million Europeans suffer from Inflammatory Bowel Diseases as of a 2009 estimate (Kappelman et al. 2013). The disease burden may in fact be much higher because the condition is not reportable and a comprehensive surveillance program has not been created, thus placing estimates at 1.5 million Americans (Long et al. 2014; Loftus 2016). An increase in age is associated with an increased prevalence of both CD and UC, with a generally greater prevalence in the Northeast and Midwest of the U.S. (Kappelman et al. 2013), suggesting environmental factors, including potentially variable bacteria across regions, may be associated with the disease. Diagnosis typically occurs in the late 20's, with monitoring and treatment required for the rest of their lives due to the chronic, uncurative nature of the diseases (Loftus 2016).

Pathogenesis

Genetic, environmental, immunoregulatory agents and the intestinal microbial environment contribute to the pathology of IBD (Sartor and Mazmanian 2012). Genetic susceptibility inferred by genome wide association studies (GWAS) suggest that the ability to sense microbes and regulate immunological responses to the environment play a role in the pathogenesis. Over 163 genes show a linkage to the development of IBD (Zhang and Li 2014). The hits of these GWAS include genes that are associated with bacterial sensing and signaling, such as NOD2 and ATG16L1 (Franke et al. 2010).

An examination of the heritability in families and in twin studies reveals that the genetic influence appears to be greater in Crohn's than in Ulcerative Colitis; the low concordance suggests that bacterial pathogens or infectious etiology may play a role (Spehlmann et al. 2008; Halfvarson et al. 2003; Orholm et al. 2000; Eckburg and Relman 2007). Gene loci account for as much as 20-25% of the disease heritability (Zhang and Li 2014), with the remaining missing heritability unexplainable. Interactions among the genes and gene-environment interactions likely promote IBD pathogenesis. One example of a gene-environment interaction (specifically, bacteria in the intestinal environment) involves CARD15. CARD15 binds MDP, a cell wall component of almost all bacteria, and a mutant CARD15 fails to clear Salmonella from epithelial cells (Sartor 2006). Other genetic susceptibilities can render the epithelium more likely to be compromised, allowing for bacterial invasion. Genetics may also affect the immune system's capacity to respond to the environment. Environmental risk factors include smoking, diet, drugs, geography, social and psychological stress (Zhang and Li 2014), and weakened immune systems of genetically susceptible people may provide the perfect storm for IBD development. Associations with the geographic regions of the north, industrialized countries, "cleaner" or more sanitized lifestyles, white collar occupations, a western diet, and prior smoking suggest that microorganisms in these environments may contribute to the dysbiosis (altered microbial composition) present in IBD patients and thus the pathology (Loftus 2004). There is a decreased risk for IBD by lowering intake of certain fats and meats, and it is generally recommended to increase consumption of fruit and vegetables, with higher omega-3 fat than omega-6 fat as part of the diet (Forbes et al. 2017). Other preventive measures include intake of vitamin D

and zinc to avoid CD and breastfeeding (Forbes et al. 2017). Also, the diet may alter the microbiome and thus contribute to disease pathogenesis as has been suggested by animal models (Devkota et al. 2012). Thus, the genetic, immunoregulatory mechanisms, and dietary influences all associate with enteric microbial contributions to the disease progression, demonstrating that the bacteria play a major role in IBD pathogenesis.

Bacterial Associations with IBD Pathogenesis

Possible mechanisms of the bacterially-associated development of IBD include infections that persist, dysbiosis (altered distribution of microorganisms, in which pathogenic ones potentially predominate or more actively influence the luminal environment), a compromised mucosal barrier and inability to clear microorganisms from the intestine, and dysregulated immunological responses, such as an overreactive T-cell response to commensal bacteria (Triantafillidis et al. 2011). Pathogenic bacteria, that is, bacteria that may cause the actual disease of IBD, or dysbiotic communities of bacteria (certain imbalances of types of commensal bacteria or commensal bacteria behaving in a different way) have been implicated in IBD development. First of all, bacteria is necessary for the immunocompromised mouse model, $IL10^{-/-}$ to develop spontaneous colitis (Sellon et al. 1998). Specifically, it has been shown that the bacterium *Helicobacter hepaticus* induces more severe colitis in $IL10^{-/-}$ (Kullberg et al. 2001). Additional studies in animal models demonstrate that losing the ability to sense intestinal bacteria through loss of signaling pathways using specific toll-like receptors (TLR), such as TLR4 (Fukata et al. 2005), myeloid differentiation primary response protein MyD88 (Araki et al. 2005), or nucleotide oligomerization domain-1 (Chen et al.

2008) or -2 (Watanabe et al. 2006) (NOD-1 or -2) leads to more severe colitis under Dextran Sodium Sulfate (DSS) inducement. This suggests that a compromised mucosal barrier and lack of sensing the microorganisms, which may lead to not clearing the microorganisms from the intestine, contribute to the disease. Pretreatment with bacterial components such as muramyl dipeptide, MDP (Watanabe et al. 2008) or lipopolysaccharide, LPS (Im et al. 2011) ameliorates DSS-induced colitis. Educating the immune system or tolerization may be the mechanism by which these pretreatments prevent the inflammatory response. Thus, animal studies support the model that specific bacterial mechanisms contribute to colitis development.

Human studies have been unable to pinpoint a causative pathogen, though many associations have been made. Initially, Dalziel's description of Crohn's in 1913 suggested that infection might be a cause, since he compared it with Johne's disease, a disease which afflicts cattle and develops by infection with *Mycobacterium Avium Paratuberculosis* (Hansen et al. 2010). The infectious organisms *Salmonella* and *Campylobacter gastroenteritis* are associated with a higher risk in the short- and long-term for developing IBD (Gradel et al. 2009). The disrupted community of bacteria, or the intestinal dysbiosis, may be a complex combination of various pathogenic bacteria such as *Escherichia coli* (*E. coli*) and other *Enterobacteria* dominating a smaller population of beneficial strains, such as *Bifidobacteria*, *Lactobacilli* and *Firmicutes* (Chassaing and Darfeuille–Michaud 2011). On the other hand, the same data suggests that bacteria, or certain types, are protective. First of all, populations showing higher IBD incidence and lower rates of reportable enteric infection indicate an association that exposure to bacteria protects against IBD development (Green et al. 2006). This

phenomenon is called the “hygiene hypothesis.” In fact, *Helicobacter pylori* is apparently protective because *H. pylori* infection seems to be associated with a risk reduction of developing IBD (Rokkas et al. 2015), complicated by the possibility that, upon detection of *H. pylori*, the bacteria was subsequently eradicated and thus the antibiotics prevented IBD (Papamichael et al. 2014). While antibiotics show some efficacy in improving symptoms, antibiotics do not cure IBD. While fecal diversion itself does not prevent recurrence of the disease, combining it with medication and localized treatment may improve healing for Crohn’s Disease (Geltzeiler et al. 2014). Because inflammation may resolve during fecal diversion but can be reactivated once the fecal stream contacts the intestine again (Rutgeerts et al. 1991), altering the bacterial composition can be beneficial for mitigating IBD. In fact, fecal microbial transplantation, which transfers microbial communities from healthy individuals or healthy-defined bacterial populations to diseased individuals’ intestines, ameliorates some cases of IBD (even though recent larger scale trials have not yet been able to prove more global efficacy of FMT for UC) (Lopez and Grinspan 2016; Borody et al. 1989; Bennet and Brinkman 1989).

Infections or dysbiosis that trigger IBD development are examples of bacteria acting at the first step, leading to intestinal permeability and staying in the intestine to perpetuate the disease. The pathogenic bacteria play an initiating role in the following three-stage model of IBD development (Sewell et al. 2009). First, the model describes the intestinal wall invaded by the bacteria and other material from the lumen, either due to viral or bacterial infection or a weak mucosa/more permeable intestinal epithelium. Hypoxia, trauma, high pressure inside the lumen, and non-steroidal anti-inflammatory

drugs may also be causes of rupturing or damaging the intestine at this initial stage. In the next stage, the damaged intestinal wall cannot remove the foreign debris completely. This stage involves pattern recognition receptors that identify the bacterial components, the action of macrophages that release pro-inflammatory cytokines, and the accumulation of neutrophils which is insufficient to rid the protective intestinal barrier of the foreign material. The adaptive immune system is activated and excessive cytokines signal downstream. The barrier of intestinal cells that protects the host from its environment is compromised, allowing bacterial antigens access deeper into the mucosal layer. Finally, the third stage of the immunological response progresses from the molecular signaling to the diseased tissue. If the number of neutrophils is insufficient to fight off the foreign material, excess debris remains and macrophages phagocytose this debris. To quarantine this invasive material and protect the host, the cells form granulomata, the inflamed tissue of the disease. This “compensatory adaptive immune response” secretes excessive pro-inflammatory cytokines and the intestinal surface appears reddened and ulcerated upon inspection by the gastroenterologists (Sewell et al. 2009).

Under normal circumstances, the innate immune system will cause cells to defend against bacterial signals; abnormal immune responses trigger aberrant signaling that dysregulate the inflammatory response (Triantafillidis et al. 2011). Toll-like receptors (TLRs) on the surface of the intestinal epithelial cells sense the bacteria through interaction with the toll-like ligands. Several bacterial components and metabolites are toll-like ligands that bind to specific TLRs. The majority of the TLRs channel through one protein, MyD88. MyD88 activates NF-kB and many downstream inflammatory cytokines

and markers (Sartor 2006). Sensing bacteria and responding appropriately allows the host to setup proper defense systems and to live symbiotically with commensal bacteria that dominate the intestine (after all, the bacterial cells have been reported to be present at 10^{10} bacteria/gram of feces (Savage 1977)). For example, mice that do not have MyD88 in their intestine and mice raised without bacteria in their intestine develop worse colitis induced by the chemical dextran sodium sulfate, DSS (Rakoff-Nahoum et al. 2004). Thus, either pathogenic bacteria, genetically-susceptible/compromised individuals, or improper immunological responses or a combination contribute to the inflammatory bowel disease pathogenesis from dysfunctional activity.

IBD Therapies

Due to the complexity of factors contributing to IBD as well as the lack of a complete cure, patients resort to various treatment strategies over the course of their lifetimes. For the chronically ill patient, the goal is often to prevent and to mitigate flare-ups, so some long-term drug programs may assist while acute treatments may help during the actual episodes of pain. If the disease has progressed to sufficient tissue destruction that is unmanageable by drug treatments to reduce inflammation and prevent constant pain, then more drastic measures such as intestinal surgery may be needed. The ideal would be to maintain healthy intestinal cells, which is often described as “mucosal healing” as a therapeutic endpoint. While mucosal healing is not clearly established, the basic working definition is “the complete resolution of the visible alterations or lesions, irrespective of their severity and/or type at baseline colonoscopy” (Neurath and Travis 2012). Endoscopic examination and judgment by eye is required.

Sometimes it has been attempted to be quantified with a Crohn's Disease Endoscopic Index of Severity (CDEIS), and for Ulcerative Colitis, the UC Disease Activity Index or other scales (Peyrin-Biroulet et al. 2016) although validation and consistent usage of a standard scale is lacking (Pagnini et al. 2014). Mucosal healing, though validated for CD and not for UC, has been correlated with positive long-term outcomes for patients, such as lower hospitalization rates, longer clinical remission and reduced numbers of surgical resections (Neurath and Travis 2012; Dave and Loftus 2012).

The standard treatments for IBD utilize rehydration and nutrition for the acute needs of the often malnourished patient (Semrad 2012) and immunosuppressive drugs for typical medical management of pain and inflammation, with surgery necessary in the most extreme cases (Triantafillidis et al. 2011). In the acute situation, patients may need rehydration and nutrition, as their damaged intestine has failed to absorb nutrients and/or drugs have impaired nutrient absorption; at the same time, the altered microbial environment of the intestine, due to surgery or inflammation or drugs, may impact nutrient absorption and thus parenteral nutrition may also be of benefit perioperatively (Schwartz 2016). Prior to any drug treatment, it is first important to assess for any malnutrition and ensure that the patient has sufficient iron and treatment for iron deficiency anemia is strongly recommended as first-line treatment, whether oral or IV is appropriate depending on tolerance (Forbes et al. 2017). Additionally, micronutrient deficiencies may exist, especially for those with diseased small intestinal tissue which absorbs the nutrients and due to loss of nutrients through diarrhea and inadequate food intake due to anorexia from active disease (Forbes et al. 2017). While the general consensus is that IBD patients benefit from a healthy diet that would be recommended

to the general public, there is a strong recommendation to increase protein from 1 to 1.2-1.5 g/kg/day during active IBD to prevent lean muscle loss and adiposity increase (Forbes et al. 2017). Enteral nutrition therapy may be the first-line option in certain cases of adults with CD, and there are many specific nutritional considerations for taking certain drugs in order to avoid certain types of nutrient or vitamin deficiencies (Forbes et al. 2017). Thus, it is important to monitor the nutritional state of the patient constantly.

Immunosuppressive drugs typically are used to avoid the more drastic measure of surgery. For ulcerative colitis, 20 to 30% of patients over the course of 10 years require a colectomy usually due to dysplasia or malignancy, which can be predicted based on inflammation and steroid use (Hefti et al. 2009; Langholz 2010; Allison et al. 2008). For chronic management of the disease, drugs are often necessary to mitigate the pain and difficulty in daily functioning due to bleeding, diarrhea, and abdominal aching. Medications attempt to reduce the inflammatory bowel disease course and allow the intestine to heal. At the same time, these drugs often include systemic disruption resulting in many negative side effects.

Conventional drug therapies dominate the drug treatments, while the use of biologics is growing in popularity for more severe stages of IBD (Triantafillidis et al. 2011; Danese et al. 2015). The majority of drugs act in an immunosuppressive manner, targeted to prevent the inflammation and subsequent cell death mechanisms. Mesalazine is one of the most commonly used aminosalicylates (although it is not actually FDA approved for UC (Sandborn 2016)) for remission of mild to moderate active UC (Triantafillidis et al. 2011) and may sometimes achieve mucosal healing

(Neurath and Travis 2012) but not considered very effective (Sandborn 2016). If renal function deteriorates, aminosalicylates must be stopped. Mesalazine is not indicated for CD (Triantafillidis et al. 2011). Patients may need to switch from mesalazine or other aminosalicylates to corticosteroids. Corticosteroids suppress inflammation and downregulate the cytokine response; they are considered highly effective as initial therapy for both UC and CD, mainly for moderate to severe flare-ups, yet one-third of patients fail to respond, and long-term use is not recommended due to toxicity concerns (Triantafillidis et al. 2011; Bernstein 2015). Corticosteroids, specifically budesonide, also appear to achieve mucosal healing in UC (Neurath and Travis 2012) and CD (Dave and Loftus 2012). The immunosuppressive drug class inhibits lymphocytes from proliferating and activating further immune responses and have demonstrated efficacy in inducing and maintaining remission of IBD. Commonly-used major immunosuppressives include azathioprine or 6-mercatopurine (although this use is off-label (Sandborn 2016)), which are considered to be effective in approximately 40% of IBD patients after 5 years; however, adverse events have led 25% of patients to stop treatment before 3 months of use and healthcare providers need to monitor serum levels (Triantafillidis et al. 2011). One of the thiopurines, azathioprine, has been demonstrated to achieve mucosal healing (Neurath and Travis 2012). Methotrexate, chemotherapy used for cancer, is also often used for UC but the larger scale randomized trials for that indication remain to be completed (Bernstein 2015). Patients may need to switch to a drug of the same class or a different class when primary drugs do not work. Thiopurines also maintain CD and UC remission but require monitoring of the serum thiopurine methyltransferase levels to ensure that the drug is metabolized and cleared from the body; other risks include non-

Hodgkin's lymphoma and nonmelanoma skin cancer (Bernstein 2015). The biologics in particular create high costs for the healthcare system; these include anti-Tumor Necrosis Factor (TNF) agents (infliximab, certolizumab pegol, adalimumab and golimumab) and anti-integrin molecules (natalizumab and vedolizumab) (Danese et al. 2015). Anti-TNF agents appear to be effective in inducing mucosal healing (Neurath and Travis 2012). Specifically, infliximab was demonstrated to be more effective than adalimumab in inducing clinical response and mucosal healing, but only through a network data analysis, while head-to-head trials of the biologic agents are still needed (Danese et al. 2015). The biologics are not effective in up to one –third of patients and are quite expensive (Bernstein 2015). Within a year of treatment, 45-60% of patients no longer receive benefit from these therapies (Triantafillidis et al. 2011; Gordon et al. 2015); sometimes patients may develop antibodies to the drugs (Sandborn 2016). Corticosteroid-free clinical remission, correlated with mucosal healing, is also considered an endpoint for amelioration of IBD (Triantafillidis et al. 2011). Thus, while several of these drugs appear to be effective, particularly towards the endpoint of mucosal healing, side-effects and loss of effectiveness over time requires alternative or new treatment options for many patients.

IBD Therapies Targeting the Intestinal Bacterial Community

Therapies for IBD have also attempted to target the bacterial aggravation of colitis by using antibiotic or probiotic therapy. Since the colon harbors much more bacteria than the ileum, it is likely that bacterially-targeted therapies are more significantly

effective in colonic disease such as ulcerative colitis. Antibiotics typically are used, not only since their use is widespread in the hospital to prevent infection, but also as an adjuvant/adjunct type of therapy in IBD. Antibiotics are believed to add a significant benefit in UC. Many utilize antibiotics as adjuvant and even first-line therapy even though studies have not been conclusive on their efficacy (Greenberg 2004) and the effectiveness seems to be more associated with colonic rather than ileal disease (Nitzan et al. 2016). Antibiotic use post-surgery has reduced the risk of endoscopic recurrence (de Cruz et al. 2013), suggesting some antimicrobial action may tend toward the therapeutic endpoint of mucosal healing. While antibiotics is appropriate for treating other complications of IBD such as sepsis, overgrowth of bacteria, and infections in abscesses or post-operative wounds, it has limited efficacy in maintaining remission and treating pouchitis, which is inflammation of the small intestine connected to the anus (resulting from removal of the colon in the case of severe UC that has damaged the large intestine). Additionally, risks and side effects of using antibiotics may include tendonitis, tendon rupture, cartilage growth prevention in fetuses and children, oral thrush, and photo sensitivity for ciproflaxin. *Clostridium difficile* infection (CDI) and antibiotic resistance are real threats. Recurrent, intractable CDI requires a fecal microbial transplantation, FMT (Leffler and Lamont 2015), and antibiotic-resistant bacterial overgrowth maybe treated via other antibiotics or eventually lead to CDI and finally FMT. While there have been many studies on antibiotic use, the 2009 American College of Gastroenterology (ACOG) guidelines state that controlled trials have not consistently demonstrated efficacy for luminal disease but antibiotics may be appropriate for infections or abscesses. Other guidelines in Europe and Britain have

similar suggestions for using antibiotics for infections but not for the active IBD disease alone as first-line therapy (Nitzan et al. 2016).

However, the destruction of the microbiome comes with a tradeoff of the loss of beneficial bacteria. Switching to probiotics to add in beneficial bacteria is an alternative strategy, requiring a break from antibiotics for about two weeks. Beyond simply adding in healthy bacterial residents, probiotic administration increases remission rates. The two most researched probiotic supplements for use in IBD are *Escherichia Coli Nissle* 1917 and VSL#3 (Sood et al. 2009), which is a highly-concentrated formulation of bacterial strains, *Lactobacilli* (*L paracasei***,** *L plantarum***,** *L acidophilus***,** and *L delbrueckii* subspecies *bulgaricus***),** strains of *Bifidobacteria* **(***B longum***,** *B breve***,** and *B infantis***),** and *Streptococcus thermophiles* (Chapman et al. 2006)**.** Non-pathogenic *E. Coli Nissle* 1917 sustained UC remission with efficacy equivalent to mesalazine (Kruis et al. 1997). VSL#3 has been demonstrated both to induce and to maintain remission for UC (Orel and Kamhi Trop 2014). Probiotic usage, such as VSL#3, for remission of Crohn's Disease has not demonstrated significantly different outcomes than placebo after surgery (Triantafillidis et al. 2011; Fedorak et al. 2015).

Another way to affect the microbial population of the intestine is by prebiotics, food components that pass through the upper gastrointestinal tract undigested and may be processed by colonic bacteria. *Bifidobacteria* and *lactobacilli* metabolize carbohydrates in this category, including oligofructose, inulin and galacto-oligosaccharides. Fructooligosaccharides, (FOS), fermentable by *Lactobacilli*, have been studied in CD without demonstrating benefit (Benjamin et al. 2011). Few studies have been conducted for prebiotics in CD and UC (Bernstein 2015). Thus prebiotics are not currently

indicated, but could be beneficial, most likely for UC as there are more bacteria in the colon.

While diet is known to alter the microbiome, both diet and microbiome remain to be explicitly determined in composition for the treatment or assessment of remission of IBD (Albenberg et al. 2012). Some diets that take a balanced approach to nutrition may also be potentially therapeutic, but research is still needed. For example, the low fermentable oligosaccharide, disacchardide, monosaccharide, and polyol (FODMAP) diet emphasizes a reduction in carbohydrates that are not absorbed well and that bacteria in the colon would metabolize (ferment), and it has been demonstrated in a randomized, controlled, cross-over trial to alleviate irritable bowel syndrome, abdominal pain, bloating/gas, diarrhea or constipation without evidence of inflamed intestine that is observed in IBD (Halmos et al. 2014). Small pilot studies show promise for FODMAP use in IBD patients (Knight-Sepulveda et al. 2015). Another potentially efficacious diet is the IBD-AID, the Inflammatory Bowel Disease – Anti-Inflammatory Diet, which modifies specific carbohydrates, promotes prebiotics and probiotics, decreases total and saturated fats while increasing omega-3 fats, adjusts food texture for improved absorption and less intact fiber and customizes dietary consumption for nutrient inadequacies, food intolerances, and food triggers (Knight-Sepulveda et al. 2015). Controlled, long-term trials on the impact of diets such as FODMAP and IBD-AID are needed to assess mucosal healing and changes in gut microbiome as well as any nutritional inadequacies (Knight-Sepulveda et al. 2015).

Currently, the most effective therapies for IBD may promote mucosal healing, the therapeutic endpoint, which can be achieved in 5-44% for CD patients (de Cruz et al.

2013) or around 80% for both UC and CD based on more recent data (Cintolo et al. 2016). At the same time, this high success rate is short-lived; in less than a year, up to 50% of those who achieved mucosal healing experience clinical relapses (Cintolo et al. 2016). The treatments come with costs both to physical and financial health: the risk of opportunistic infections for both antibiotic use and the immunosuppressive drugs, the malignant side-effects of biologics, and the high costs of drugs, particularly that of expensive biologics (Sandborn 2016). It remains unclear as to how and when to de-escalate drug regimens and which drug type to attempt next for therapy (Cintolo et al. 2016). While the majority of treatments target the immune response, newer treatment options would be desirable to minimize negative impacts on the immune system or to promote beneficial bacterial populations in order to assist in mucosal healing. Natural products or drugs that have proven safety profiles for other diseases are worth investigating. For example, repurposing anti-oxidants, statins, rosiglitazone for IBD have shown promise in preliminary studies and small clinical trials and remain to be studied further (Triantafillidis et al. 2011). To advance these new possible therapies, the field needs a greater mechanistic understanding of the beneficial effects of bacterial populations and the potential cell signaling pathways to exploit for promoting intestinal epithelial cell health.

The Role of HMGB1 in IBD

Extracellular HMGB1 in IBD

Although High Mobility Group Box 1 protein (HMGB1) does not show up in the GWAS (Franke et al. 2010), HMGB1 has been implicated in several studies of IBD models (Harris et al. 2012) and warrants further investigation for developing IBD therapies. HMGB1 is a highly-conserved, ubiquitously expressed protein that resides predominantly in the nucleus, but is present at relatively high extracellular levels in the feces of IBD animal models and of pediatric IBD (Harris et al. 2012; Vitali et al. 2011). Little investigation was performed regarding its nuclear role in maintaining genomic integrity, regulating gene transcription, aiding DNA repair and recombination before its extracellular role became popular (Hu et al. 2015). Usually extracellular HMGB1 has been examined in the context of immune cells, and may be passively released from necrotic cells or actively secreted by stimulated cells such as macrophages (Hu et al. 2015). HMGB1 has mainly been described as a cytokine or danger associated molecular pattern molecule, triggering inflammation through binding to RAGE, TLR2 or TLR4 and stimulating more cytokine release (Lotze and Tracey 2005, Hu et al. 2015). However, this recombinant HMGB1 tested in these models was produced in bacteria, and residual bacteria may have contributed to the aggravation of inflammation that recombinant HMGB1 produced in various inflammatory disease mouse models. Targeting HMGB1 for therapy has been attempted through removing HMGB1 using antibodies, beads (Ju et al. 2013) or other mechanisms (Andersson and Tracey 2011). Thus extracellular HMGB1 has been targeted for removal as a therapy, but its intracellular role remains less well understood.

Before HMGB1 is secreted or released to function in this extracellular role, HMGB1 is present in the cytosol, either post-translation or post-export from the nucleus.

Posttranslational modifications such as acetylation, phosphorylation, methylation, ADP ribosylation or redox modification may determine different conformations of HMGB1 that affect its functions extracellularly or in nuclear or cytosolic compartments (Hu et al. 2015). Bacterial component Lipopolysaccharide apparently induces acetylation of HMGB1 and prevents its nuclear import in monocytes (Bonaldi et al. 2003), which perhaps allows it to be retained and function cytosolically when needed in those conditions. Export from the nucleus may occur under certain conditions. Under stress - induced by microbes or nutrients - HMGB1 rapidly translocates from its main residence in the nucleus to the cytosol (Tang et al. 2010), and it is this cytosolic function that is not as intensively studied. Intracellular HMGB1 has a major role in the whole organism, and likely in metabolism or glucose regulation, since global knockout mice (HMGB1-/-) die a few days after birth due to hypoglycemia and tissue atrophy (Calogero et al. 1999). While much of the literature on HMGB1 has studied its role in immune cells and neuronal cells, less has been investigated in intestinal epithelial cells. Recently, its intracellular role in the intestine has been investigated through the use of a model with an intestinal-specific knockout of HMGB1, the Vil-Cre Hmgb1fl/fl mice. This model allows for the investigation of intracellular HMGB1 in the intestine.

Intracellular HMGB1 in IBD

Cytosolic HMGB1 functions in a protective manner, switching between autophagy and apoptosis under microbially-induced inflammation (Zhu et al. 2015). This recent work showed that mice deficient in intestinal epithelial cell (IEC) HMGB1 developed

worse colitis in the genetic and chemically-induced mouse models (induced by IL-$10^{-/-}$ and DSS) (Zhu et al. 2015). As a human counterpart to these studies, HMGB1 expression in human biopsies showed lower protein levels in the intestinal samples from patients with active IBD relative to protein levels in controls' samples (Zhu et al. 2015). Since bacteria plays a major role in IBD, and may be a source of stress to induce HMGB1's translocation from the nucleus to the cytosol, the bacteria's effect was tested *in vitro*. Bacteria induces intracellular HMGB1 expression. Without HMGB1, the cells do not respond well to bacteria. Under bacterial stress, HMGB1-deficient IEC demonstrated elevated levels of calpain activity, which is pro-inflammatory, and inflammation-induced death. Therefore, the bacterial regulation of HMGB1 and its role as an intracellular protective shield from cell death present a new opportunity for investigation into IBD therapies involving the activity of HMGB1.

IBD Therapies Exploiting HMGB1's Intracellular Role

Since HMGB1 is protective but also expressed at lower levels in the intestinal mucosa of IBD patients, possible therapeutic strategies could either 1) raise the protective HMGB1 protein levels in the intestine of patients with active disease (restoring HMGB1 to sufficient levels for its protective intracellular role, and using HMGB1-dependent cellular defenses), or 2) bypass the need for HMGB1 altogether, utilizing a treatment that activates the protective function of HMGB1 in HMGB1's absence (an HMGB1 independent therapy).

In the first place, since bacterial regulation may promote HMGB1 expression, identifying other bacterial factors that could promote HMGB1 provides more knowledge regarding the fine-tuning of appropriate levels of intracellular HMGB1 expression in its stress-response function as a cytosolic protein. While muramyl dipeptide, MDP, a common component of the cell wall of many gram-negative and gram-positive bacteria, induced HMGB1 expression (Zhu et al. 2015), it is possible that other microbial factors may induce HMGB1 expression as well through similar mechanisms. The promoter region for the HMGB1 gene contains a site that can bind the transcription factor activator protein (AP)-1 (Fink 2007). Since MDP activates nucleotide oligomerization domain (NOD)-2 (Barnich et al. 2005) and Toll-Like Receptor (TLR) 4, (Strober et al. 2006) and both those receptors signal through MyD88, which can activate AP-1 (Medzhitov et al. 1998), HMGB1 is potentially regulated through MyD88. Thus, other bacterial components sensed through TLRs which signal through MyD88 may also stimulate HMGB1 expression.

Of the possible candidate bacterial components that would stimulate intracellular HMGB1, those that promote intestinal epithelial cell health or decrease inflammation would be applicable. Other cell wall components besides MDP include lipopolysaccharide (LPS), lipoteichoic acid (LTA), CpG DNA (bacterial DNA mimic), and flagellin (FLA). MDP treatment has been shown to promote the function of the epithelial barrier and maintain homeostasis of the intestine by sustaining epithelial integrity in mouse colonic epithelial cells (Hiemstra et al. 2012). Additionally, MDP treatment of intestinal organoids promoted growth of the IEC (Nigro et al. 2014), treated at the same concentration as intestinal organoids that demonstrated MDP promoted HMGB1

expression (Zhu et al. 2015). Increased cell viability accompanying increased cytosolic HMGB1 would theoretically lead to IEC mucosal healing. Furthermore, MDP treatment in a mouse model ameliorated trinitrobenzenesulfonic acid (TNBS) and DSS-induced colitis (Watanabe et al. 2008). Analogously, LPS, lipopolysaccharide or endotoxin from gram-negative bacteria (Makimura et al. 2007), may be beneficial in the treatment of colitis as well, through HMGB1 protection. LPS, like MDP, has been associated with decreased DSS-induced colitis (Im et al. 2011). At the same time, research in Caco-2 cells showed that LPS administration led to excessive apoptosis (Suzuki et al. 2003). Similar administration of LPS in organoids alternatively led to cell growth (Nigro et al. 2014). Thus it is important to investigate the role of LPS in primary epithelial cells at an appropriate concentration and duration to understand its role in HMGB1 promotion and cellular viability. Lipoteichoic acid, LTA, from gram-positive bacteria cell walls may be purchased as isolated from pathogenic strains such as *Staphylococcus aureus* or probiotic strains such as *B. subtilis*. LTA-deficient *L. acidophilus* mitigated DSS colitis (Mohamedzeh et al. 2011) and *S. aureus*-derived LTA induced pro-inflammatory signals and subsequent colitis in a DSS model (Zadeh et al. 2012), which demonstrate the inflammatory type of LTA. On the other hand, *L. plantarum* –derived LTA prevented inflammation in HT-29 cells (Kim et al. 2012) and *B. subtilis*-derived LTA promoted epithelial barrier function in porcine epithelial cells (Gu et al. 2014), showing that some LTA may be anti-inflammatory and benefit intestinal epithelial integrity. Thus, *B. subtilis*-derived LTA was used in this study for its effect on HMGB1 (predicted based on its signaling through TLR2 (Cabral et al. 2013)) and promotion of IEC viability. Bacterial DNA, which may be represented by a higher frequency of sections of unmethylated

CpG islands of DNA that renders it distinct from mammalian DNA, may activate the immune system by binding to IEC TLR9 (Weiner et al. 1997; Pedersen et al. 2005). While CpG DNA (or CpG ODN, for oligodeoxynucleotides) has been shown to exacerbate colitis in a DSS animal model (Obermeier et al. 2002), it could also stimulate HMGB1's promoter AP-1 (Min et al. 2012). At the same time, since HMGB1 is a known DNA-binding protein, it has also been investigated as a CpG-ODN-binding protein. Its multifunctional nature may allow HMGB1 to bind CpG-DNA in the nucleus, cytosol, or extracellularly, complicating the dynamic nature of its relationship to autophagic and apoptotic mechanisms (Ivanov et al. 2007). It remains to be determined as to whether CpG DNA treatment of primary IEC would promote HMGB1 levels in the cytosol to a sufficient degree to sustain IEC health and be protective against inflammation-induced cell death. Flagellin, a TLR5 ligand, stimulates inflammatory responses in IEC (Gewirtz et al. 2001), immune modulation in HT-29 cells (Dobrijevic et al. 2013) and increased intestinal barrier permeability in T-84 cells (Oppong et al. 2013). Thus, flagellin is a potential candidate to promote HMGB1 and intestinal epithelial health as well. These components and their pathways to induce expression of HMGB1 are depicted in Figure 1.

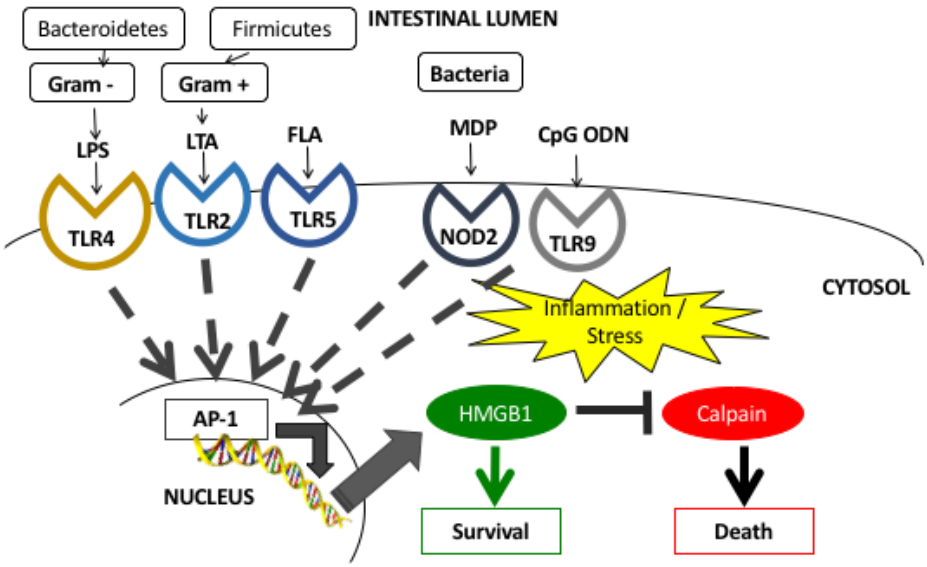

Figure 1: Various bacterial components may induce expression of HMGB1.
This hypothetical model shows how various bacterial components which are associated with AP-1 activity could promote expression of HMGB1, which acts in the cytosol to modulate calpain, thereby promoting cellular survival.

As for the second approach to intracellular HMGB1-informed IBD therapy, promoting IEC health in an HMGB1-independent manner requires knowledge of the mechanism of HMGB1's protective function inside the IEC. Thus, not only does the following study aim 1) to identify bacterial regulators of HMGB1, but more importantly, 2) to treat HMGB1-deficient cells under bacterial stress, to promote IEC viability with the goal of promoting mucosal healing for IBD patients. While the previous work showed that mRNA of HMGB1 was elevated at the same time as HMGB1 protein was depressed in active IBD lesions (Zhu et al. 2015), it is unlikely that activating the HMGB1 gene promoter will translate to increased cytosolic HMGB1 protein. Candidate drug treatments for this scenario need to bypass the need for HMGB1 to protect calpain-client proteins from being cleaved and thus prevent excessive inflammation

leading to apoptosis, which was demonstrated in the *Vil-Cre Hmgb1*$^{fl/fl}$ mice previously (Zhu et al. 2015). Therefore, not only does this study investigate bacterial regulation of HMGB1 and HMGB1-dependent bacterial-stress induced death, it also investigates a mechanism by which HMGB1 may promote cell viability and then identifies candidate drugs that rescue cell health when HMGB1-deficient cells face bacterial stress.

Finally, this study utilizes the unique model of enteroids, which are mini-guts, three-dimensional structures of intestinal epithelial cells derived from primary tissue; in this case, from mice. The strength of this unique model is that it models more directly the primary intestinal epithelial cells and reduces the focus of the study to the immediate, innate immune response, not the deeper immune cells nor transformed cancer cells. Therefore, this study investigates the bacterial regulation of HMGB1, the HMGB1-dependent bacterial-stressed cell death, the mechanism of HMGB1's protection against cell death under bacterial stress, and repurposes approved potential candidate drugs for rescuing HMGB1-deficient IEC from bacterial-stress induced cell death in enteroids.

Chapter 2: Methods

Mice

HMGB1$^{f/f}$ and HMGB1 $^{f/f\ vil\text{-}CRE}$ mice were available in the lab. The mice were generated on C57B/6 mice using Ozgene (Ozgene Pty Ltd, Australia). LoxP sites flank exons 2 and 3 of the HMGB1 gene in the HMGB1$^{f/f}$ mice. The 5' untranslated region, the start codon, and the coding sequence for the first 49 amino acids of the protein are exon 2. The coding sequence for the amino acids 50-98 are contained in exon 3. The Vil-CRE mice, originally from Sylvie Robine (Genesis, 2004), were bred to the HMGB1$^{f/f}$ and resulted in generating HMGB1 $^{f/f\ vil\text{-}CRE}$ mice. By deleting the first amino acids of the protein through elimination of those two exons, the translation terminates early due to the open reading frame shift. Genotyping was performed using the primers 5'-GAGGCCTCCGTGAGTATGAP-3' and 5'-TTGCAACATCACCAATGGAT-3'.

Wildtype or *Hmgb1$^{fl/fl}$* and *Vil-Cre Hmgb1$^{fl/fl}$* C57Bl/6 mice were raised in standard husbandry conditions in a gnotobiotic (called germ-free, or GF in this study) or conventionally-raised (called specific pathogen-free, or SPF) facility to 6-18 weeks of age before utilization. The Institutional Animal Care and Use Committee (IACUC) at the University of Chicago received the submission and approved the animal use protocols for this study. The researchers performed the protocol under the committee's approval. Euthanasia was completed by asphyxiation by carbon dioxide or anesthetic with secondary means of death by cervical dislocation.

Organoid Culture

Organoids, or 3-D cultures of primary cells, were generated from isolated crypts of small intestine (termed “enteroids”). The crypts were isolated in a method as previously described (Zhu et al. 2015). Briefly, the distal ileum 10 cm above the cecum was sliced into 1 mm pieces with a razor blade, and washed with Dulbecco's Phosphate-Buffered Saline DPBS (#14190, Life Technologies, Carlsbad, CA). Then the pieces were treated for 30 min with 2.5 mM EDTA (made in DPBS from Ethylenediaminetetraacetic Acid, Tetrasodium Salt Dihydrate, BP121-500, ThermoFisher Scientific, Waltham, MA). Intestinal crypts were washed with vigorous pipetting through Advanced DMEM/F12 (ADF) (#12634, Life Technologies) media. Finally, the crypts were filtered through a 70-micron cell strainer. Pelleted cells were resuspended in complete ADF media (ADF supplemented with the following at 1 X: L-Glutamine (#25030), HEPES buffer (used at 10 mM) (#15630) and penicillin and streptomycin (#15140), all from Life Technologies. Supplemented factors included N2 supplement (#17502, Life Technologies) at 1X; B-27 Supplement Minus Vitamin A (#12587, Life Technologies) at 1X. Additional growth factors to supplement the media included murine Epidermal Growth Factor (EGF) (50 ng/ml; # 31509, Peprotech, Rocky Hill, NJ); Noggin (100 ng/ml; #250-38, Peprotech); Jagged 1 (1 μM; #AS-61298, Anaspec, Fremont, CA); Y-27632 (10 nM; #10005583, Cayman Chemical Company, Ann Arbor, MI); and R-spondin 1 (100 ng/ml; #120-38, Peprotech)). Cells were then combined with Growth-Factor Reduced Matrigel (#356231, BD Biosciences, Franklin Lakes, NJ) at a ratio of 1:2 and plated onto 12-well or 6-well plates. Matrigel beads containing crypts and cells were allowed to solidify for

approximately 1 hour at 5% CO2 and 37°C before adding 1-2 ml growth factor-supplemented complete ADF media to each well. Enteroids were fed with growth factor supplemented media approximately every 2 days until experimentation. For each passage, collected cells were centrifuged (300 g) at 5 min, washed with media and repelleted as necessary to remove cell debris and/or excessive Matrigel, and similarly plated as in the original isolation for each passage. The passage number of the enteroids at the time of treatment was not more than 15. Prior to treatment, the density of the organoids was similar from line to line.

High Throughput Screening (HTS) Preparation

Collected enteroids (*Vil-Cre Hmgb1*$^{fl/fl}$) were washed three times with ADF media, and centrifuged at 150g for 5 minutes to remove Matrigel. Cells were pelleted and resuspended in 1 ml of pre-warmed TrypLE Express, (#12605, Life Technologies), and digested at 37 deg C for at least 15 min to single cells. 10 ml ADF media were added to quench digestion. Cells were passed through a 40 micron cell strainer prior to counting with 0.2% of Trypan blue (#15250, Life Technologies) and a hemacytometer. Cells were prepared in ADF media supplemented with N2 and B27 in Matrigel (at a ratio of 1 part cells to 2 parts Matrigel) approximately 1000 cells per well in a 1024-well plate. Where appropriate, 10 μg MDP per ml of ADF media supplemented with N2 and B27 was added to the wells.

HTS Method

Thermo Multidrop Combi (Thermofisher Scientific, Waltham, MA) dispensed the cells/media/Matrigel (7 μl/well). Plate temperature was maintained at 37 degrees in a 5% CO2 incubator during experiment. After 30 minutes passed to allow for polymerization, the instrument dispensed 75 μl of ADF media supplemented with N2 and B27 (with or without MDP) and 0.4 μl of the 10 mM drug compounds from the Prestwick Chemical Library (final concentration of drug = 48.5 μM). Treatment duration was 8 hours, after which Cell Titer Glo 3D (#G9683, Promega, Madison, WI) substrate was added at a 1:1 volumetric ratio. Molecular Devices Analyst GT (Molecular Devices, San Diego, CA) measured luminescence. The plate setup is included in Appendix 1, showing treatment and genotypes used for each well. Appendix 2 shows the drugs used for each of the four plates. There were two replicate (rep) plates run for each of the four plate layouts. “Positive hits” from the HTS were defined as drug treatments that resulted in readings 1.25 fold above the average of readings from MDP-treated HMGB1-deficient enteroid cells (controls) in two separate screening plates.

Enteroid Treatment Studies

One day prior to treatment, complete growth factor-containing media was replaced with only N2 and B27 supplemented media to avoid triggering signaling pathways and to ensure that the enteroids were in a baseline state. *Hmgb1*$^{fl/fl}$ or *Vil-Cre Hmgb1*$^{fl/fl}$ enteroids were derived from male or female mice, approximately 6-18 weeks old. The passage number of enteroids used did not exceed passage 15. The bacterial factors

were sourced from Invivogen (San Diego, CA): L-18 MDP (tlrl-lmdp), LPS-EK (tlrl-eklps), Flagellin from *Salmonella typhimurium* called FLA-ST (tlrl-stfla), Lipoteichoic Acid from *Bacillus subtilis* called LTA-BS (tlrl-lta), and ODN 1826 (tlrl-1826) (Class B CpG oligonucleotide). These factors are referred to as MDP, LPS, FLA, LTA and CpG ODN (or DNA) respectively. All experiments with MDP used a dose of 10 μg/ml. Additional doses were as listed in the figures. Treatment for enteroid experiments lasted 18 hours, unless otherwise noted (1, 2, or 4 hours of treatment cell-signaling experiments), at the various doses listed in each experiment. Replicates were performed on independent biological primary cell lines derived from individual mouse intestines. Representative western blots are shown.

For the 18-hour treatment verification of the hit, we treated with 50 μM of the Lovastatin drug (#10010338, Cayman Chemical Company). For evaluation of ATP production, enteroids were digested to single cells using TrypLE Express, a portion dyed with trypan blue counted using a hemacytometer, and plated at a cell density of approximately 20,000 cells per well in a white 96 well plate for the Cell Titer Glo 3D assay from Promega. The media with N2 and B27, without extra growth factors, was provided after the Matrigel stabilized. The subsequent morning, the cells were treated with one of the following conditions: MDP, Calpeptin (#03-34-0051, EMD Millipore, Billerica, MA), an inhibitor of calpain 1 and 2, at a dose of 1 μg/ml, 0.1% Triton X-100 (BP151-500, Fisher Scientific) for eight hours, after which the Cell Titer Glo 3D substrate was added at a 1:1 volumetric ratio. Promega Glomax Multi Detection System (Promega) was used to measure luminescence.

Western blots/Immunoblots

Cells were harvested at the timepoint specified, and using cold PBS and slow centrifuging and aspiration, supernatant and Matrigel removed. Cells were lysed in 1 X from 10 X Cell Lysis Buffer (#9803, Cell Signaling Technology, Danvers, MA) containing Complete Protease Inhibitor at 1 tablet in 10 ml (#11836153001, Roche, Indianapolis, IN) and 1 mM Phenylmethylsulfonyl fluoride (PMSF) (P-7626, Sigma-Aldrich, St. Louis, MO). Protein measurement was conducted via bicinchoninic acid assay and the remainder frozen at -80 deg C, used in Laemmli buffer 3X buffer [167 mM Tris, 8 mM EDTA, 27% glycerol, 1.3% β-mercaptoethanol, 416 mM sodium dodecyl sulfate, and 0.3 mM bromophenol blue]) was added. Samples were heated at either 60°C for 10 min or 95°C for 5 min prior to separation on 10%-15% sodium dodecyl sulfate-polyacrylamide gels depending on the protein size. The Hoefer TE22 Tank Transfer Unit was used (manual includes a reference procedure). After transfer, membranes were blocked and incubated with the appropriate primary antibody. The following antibodies were used: cleaved caspase-3 (9664) at 1:1000, Akt (9272) at 1:1000, Phospho Akt (Ser473) (9271) at 1:1000, B-actin (4967L) at 1:5000 (all from Cell Signaling Technology); HMGB1 (ab18256, Abcam, Cambridge, UK) at 1:1000; p62/SQSTM-1 (5114, Cell Signaling) at 1:1000; and Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (AM4300, Ambion, Inc., Austin, TX) at 1:5000.

Either GAPDH or B-actin was used as loading control as necessary based on the size of the target protein or its secondary antibody's specificity (anti-mouse GAPDH and Anti-Rabbit B-actin). Then the membrane was washed and incubated with the

appropriate secondary, horseradish peroxidase-conjugated goat anti-rabbit or goat anti-mouse, and developed using Amersham ECL Advance (GE Healthcare).

Densitometry was performed using ImageStudioLite. Rectangles were drawn for each band, copied to be the same size per blot, and the intensity of the signal, after background subtraction (if significant), was normalized to the area, and then normalized to the intensity of the similarly normalized loading control or total protein, as applicable.

Prism (GraphPad) software was used for statistical evaluation. Two-way Analysis of Variance (ANOVA) or one-way ANOVA was used as appropriate, with matching used when the same cell line and repeated measurements over doses were taken (ratio paired t-test used when appropriate), or unpaired Student's T-test or the one-way ANOVA with Bonferroni *post hoc* testing for multiple comparisons.

Chapter 3: Results

Microbial Regulation of Intestinal Epithelial Cell HMGB1 Expression

The interaction between the intestinal epithelium and the bacterial environment in the intestine is a critical area of study for the better understanding of inflammatory bowel disease. As previous work has demonstrated the role of intestinal epithelial cell HMGB1 to be a switch between autophagy and apoptosis (Zhu et al. 2015), HMGB1's mediation of the bacterial-host interaction may dictate the cell's ability for self-defense. Intracellular HMGB1 acts as a protective shield to prevent the cascade of apoptotic signaling in intestinal cells inflicted with colitis (Zhu et al. 2015), which is a bacterially-dependent disease (Yang et al. 2013; Büchler et al. 2012; Franchimont 2004; Garrett et al. 2010; Nishikawa et al. 2009; Machiels et al. 2013; Hotte et al. 2012; Frank et al. 2011; Fava and Danese 2011). By isolating the area of study to the intestinal epithelial cells themselves, and utilizing specific isolated bacterial factors, we have the ability to work with HMGB1-competent or HMGB1-deficient intestinal epithelial cells in a less complicated model. Wildtype or *Hmgb1*$^{fl/fl}$ mouse enteroids were used to interrogate the role of specific bacterial factors in the regulation of HMGB1. The HMGB1-deficient enteroids were used as the colitis-susceptible model. Figure 2 demonstrates that HMGB1 was not present, at the expected protein level, in the enteroids derived from *Vil-Cre Hmgb1*$^{fl/fl}$ mice.

Figure 2: HMGB1 f/f and HMGB1 f/f, Vil-CRE enteroids.
A) Western blot of HMGB1 expression in protein lysates from HMGB1$^{f/f}$ and HMGB1 $^{f/f, Vil\text{-}CRE}$ enteroids. B) Photograph of enteroid (mini, three-dimensional structures with buds grown from intestinal stem cells derived from mouse) through microscope at 10x.

Previous work demonstrated that a common bacterial cell wall component, muramyl dipeptide (MDP), upregulates HMGB1 expression in intestinal enteroids (Zhu et al. 2015). We expanded the investigation into the bacterial regulation of HMGB1 expression by examining first, intestinal protein from germ-free mice and mice raised in a specific-pathogen free (SPF) environment, *in vivo,* and second, additional bacterial factor treatment studies *in vitro*. Without bacteria, mouse intestinal protein contains less HMGB1 protein per cell (Figure 3A). Thus, in the living whole organism, bacteria are necessary to sustain physiological levels of HMGB1 in the intestine. Likely other bacterial components besides the common one, MDP, stimulate HMGB1 expression, which led to further studies *in vitro*. As shown in Figure 3B, the panel of bacterial components (selected as discussed in the introduction) stimulated HMGB1 expression to varying degrees. To ensure a chance of obtaining a response, the concentrations of the components were chosen based on a review of the concentrations used in similar studies (Nigro et al. 2014; Hall et al. 2008; Ghadimi et al. 2010) which provided more narrow targets for dose choices than the wide ranges in the specifications provided by the supplier, Invivogen.

Our *in vitro* studies mainly were based upon the previous studies in our lab at which we treated mature enteroids with L-18 MDP from Invivogen for four hours and observed the induction of HMGB1 protein. When determining the panel of bacterial factors to test in our model, we evaluated similar studies. A study (Nigro et al. 2014) conducted in intestinal organoids used similar components from Invivogen: MDP, LPS, (also at the same dose) Flagellin (10 ng/ml) and CpG DNA (1 micromolar), although it is unclear whether the exact same type was used in our experiments because there are multiple products from Invivogen. The treatment period was 4 days of culture from initially seeding intestinal crypts for the study (Nigro et al. 2014). The number of organoids increased by over fourfold from MDP treatment but LPS, Flagellin and CpG DNA did not seem to have a significant effect (Nigro et al. 2014). Since 5 μg/ml of ODN 1826 is approximately 0.8 micromolar, the concentration we used was similar to that used in the organoid study. Five days of co-culture with 1-10 μg/ml of the same ODN 1826 type of CpG DNA (Hall et al. 2008) used in dendritic cells and T cells showed a cytokine-stimulating effect, suggesting that this range of dose would be effective in our studies as well. Ghadimi et al. 2010 used CpG DNA from *Lactobacillus rhamnosus GG* (LGG) and *Bifidobacterium longum* to treat cancer cell lines and showed attenuated NF-kB signaling, suggesting anti-inflammatory effects. These may coincide with or be interrelated with HMGB1 expression through TLR9 signaling since another publication shows that CpG DNA activated AP-1, HMGB1's promoter in a human *in vitro* model (Ivanov et al. 2007). We treated mature enteroids (the intestinal stem cells had developed into the budding structures of mini-guts shown in Figure 2B, and typically had been passaged several times before treatment) for an overnight treatment of 18 hours

to provide more time for a response than our initial 4 hour study. Thus, the hypothesis was that CpG DNA would induce HMGB1, and it appeared to induce HMGB1 expression strongly in two of the three replicates, so it was further tested at multiple doses.

While LPS was not as potent of an inducer, it appeared that the synthetic bacterial DNA seemed to have a very significant effect on HMGB1 expression. However, LPS is widely studied in many cell and animal models from “physiologically relevant” to “pharmacological concentrations” (Abreu 2010; Im et al. 2011; Guo et al. 2013), particularly in sepsis models (Doi et al. 2009), and, as the typical “endotoxin” cultured when analyzing for sterility, it is a popular candidate for study as well. Therefore, these two bacterial components which elicited the extreme high and low responses (Figure 3B, C) were tested at different doses to assess whether they would induce different responses in a dose-dependent manner (Figure 4).

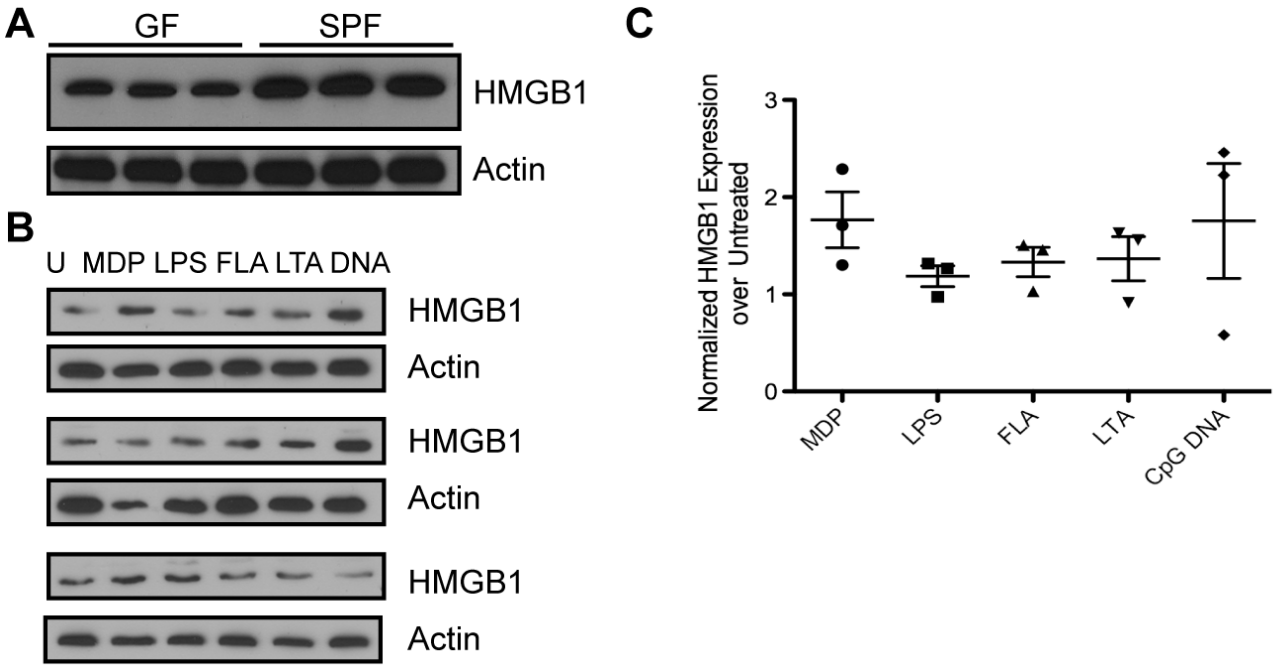

Figure 3: Microbial Regulation of HMGB1 Expression *in vivo* and *in vitro*.

(Figure 3 continued). (A) Protein from intestine of germ-free (GF) and specific pathogen free (SPF) raised mice on a western blot. B) Protein isolated from enteroids using the following treatments: U = untreated, 10 μg/ml MDP = Muramyl Dipeptide, 10 μg/ml LPS = Lipopolysaccaride, 10 ng/ml FLA = Flagellin, 10 μg/ml LTA = Lipoteichoic Acid, 5 μg/ml DNA = synthetic DNA for 18 hour overnight (Figure 3 continued) treatment. Three lines of enteroids treated with the panel of TLR ligands and assayed for HMGB1 expression. C) Quantification of HMGB1 Expression, normalized over the untreated HMGB1 expression.

Testing Lipopolysaccharide (LPS from *E. coli* K12) at 1, 10 and 30 micrograms per milliliter revealed that LPS treatment did have a dose-dependent effect. LPS induced higher levels of HMGB1 at 10 and 30 micrograms/ml, although quantification of the protein on the Western did not show any significant effects according to the statistical t-test and p-value calculations (Figure 4A). CpG DNA, the synthetic oligodeoxynucleotide ODN 1826 of unmethylated regions of CpG islands of DNA specific to bacteria rather than mammals, was more effective at the highest tested dose, 30 micrograms/ml (Figure 4B). A one-way analysis of variance (ANOVA) determined that this dose was significantly different based on a statistical p-value of 0.0279. Thus, both LPS and CpG DNA demonstrated a dose-dependence of effect on HMGB1 expression.

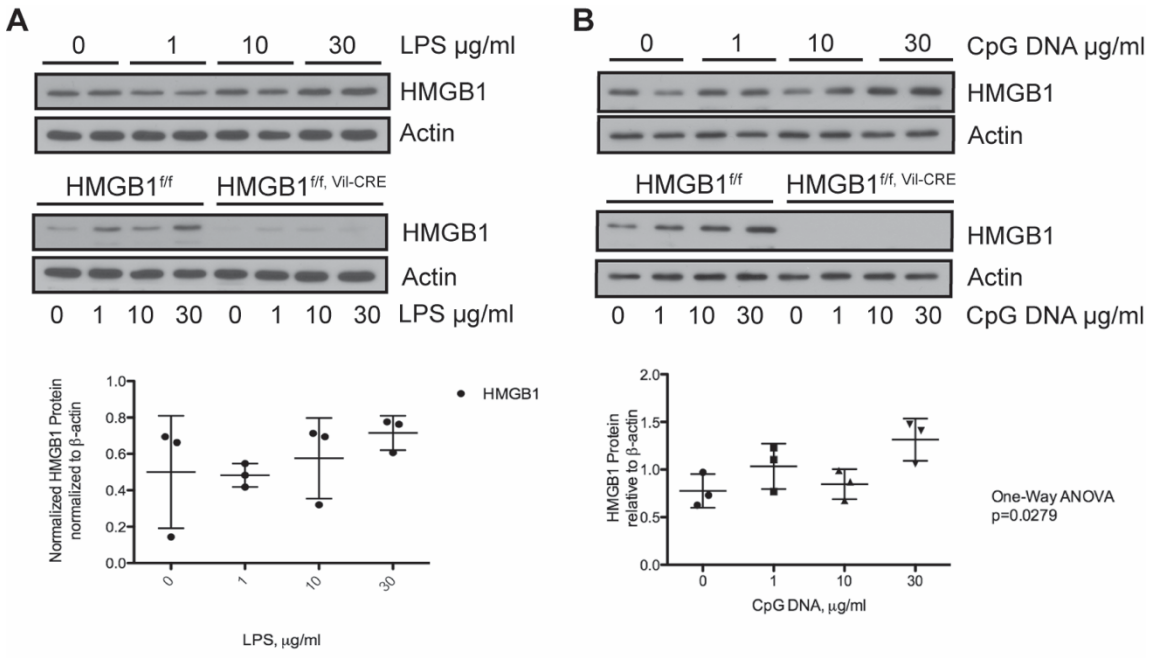

Figure 4: LPS and CpG DNA induce HMGB1 in a Dose-Dependent manner in Enteroids.
A) HMGB1 expression via western blot and quantification of western blot bands from three enteroid lines treated with doses of LPS (0, 1, 10 and 30 micrograms per milliliter). B) HMGB1 expression via western blot and quantification of western blot bands from three enteroid lines treated with doses of CpG DNA (0, 1, 10 and 30 micrograms per milliliter).

Further experiments utilized MDP and LPS since these represent the majority of bacteria, representing both gram-negative and gram-positive bacteria. To verify the effects of MDP and LPS, four different lines of enteroids, from independent mouse intestine derivations, were treated with the components and subsequently assayed for HMGB1 protein and the western blots were analyzed by densitometry (Figure 5).

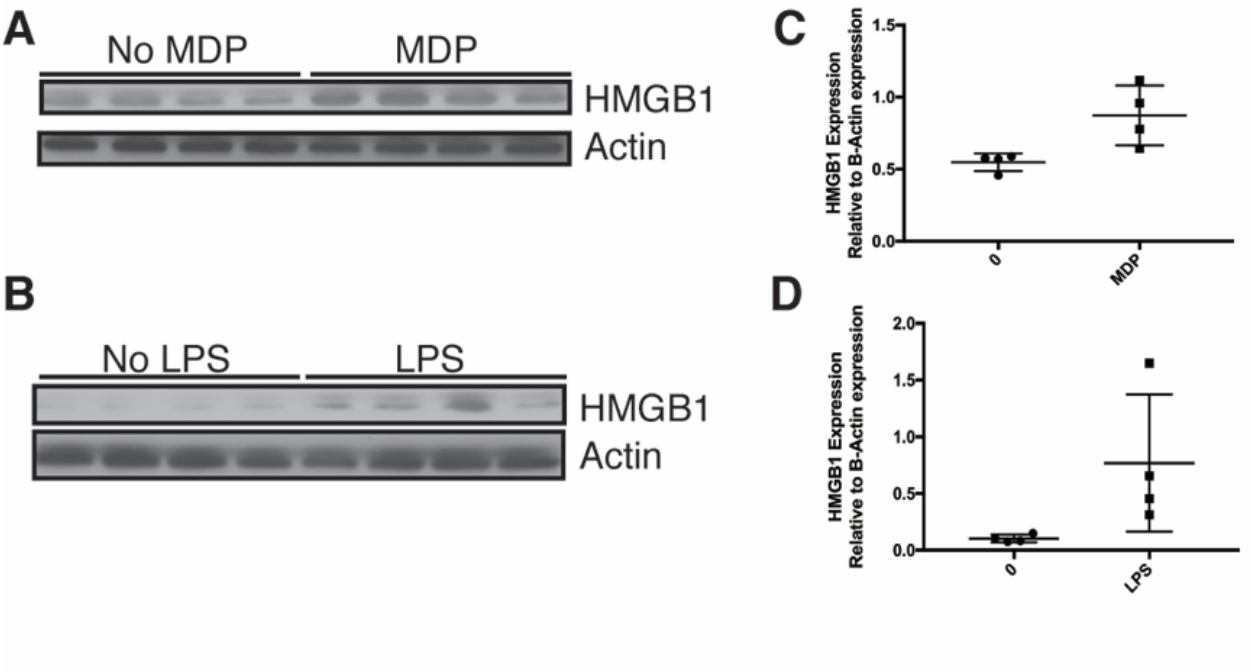

Figure 5: MDP and LPS Promote HMGB1 Expression.
A) Four lines of enteroids treated with MDP increased HMGB1 by Western blot, B) Four lines of enteroids treated with LPS show increased HMGB1 by Western blot, C) Densitometry and subsequent ratio paired t-test shows significant induction of HMGB1 by MDP treatment, p-value = 0.0379, D) Densitometry and subsequent ratio paired t-test shows significant induction of HMGB1 by LPS treatment, p-value = 0.0227.

While bacterial regulation of HMGB1's increased expression was fairly established, we did not know whether this total protein resided more in the nucleus affecting downstream transcriptomes and proteomes or the protein was active in the cytosol, protecting calpain-client proteins from further cleavage, as demonstrated previously (Zhu et al. 2015). Under stress induced by bacteria, HMGB1 translocates to the cytosol from the nucleus (Tang et al. 2010) so we hypothesized that the increase in HMGB1 that we observed was mostly due to increases in cytosolic HMGB1 where it would be active in defense. We sought to understand whether the increased HMGB1 expression was due to more cytosolic protein or more nuclear protein or both. However, when we assayed the cellular protein, we noticed that more HMGB1 was in the cytosol in the unstimulated condition (Figure 6). This showed us that our *in vitro* model likely was

stressing the cells. It appeared that more cytosolic HMGB1 was present in the stimulated conditions; however, the fact that the baseline conditions of our model do not appear to mimic *in vivo* conditions led us to leave this question for another model as our model was limited in this respect.

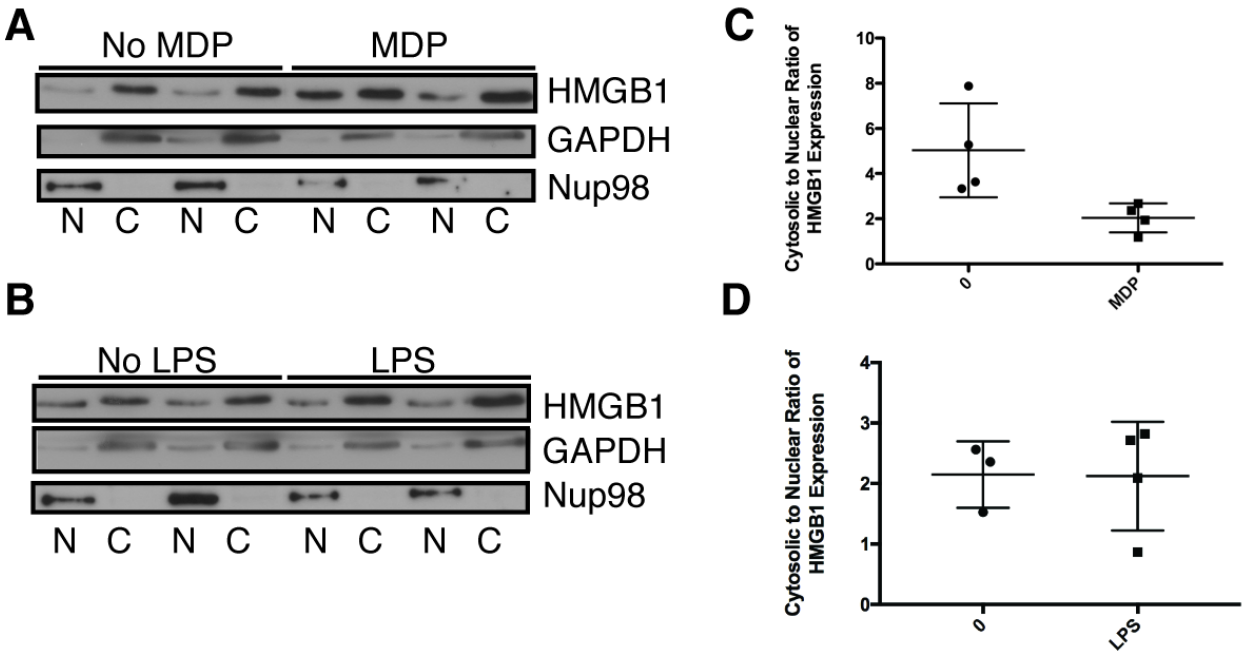

Figure 6: Bacterial Stimuli Promote Nuclear and Cytosolic HMGB1 Protein.
Nuclear and Cytosolic fractions of protein collected from A) MDP stimulated and B) LPS stimulated enteroids and respective image densitometry quantification for the C) MDP condition and the D) LPS condition.

These data demonstrate that HMGB1 protein expression in the intestine can be stimulated by various bacterial components, that the amount of the bacterial component impacts the extent to which it can affect HMGB1 protein expression and that the stimulation of HMGB1 is consistently repeatable by bacterial components MDP and LPS.

HMGB1 Cytoprotection Against Bacterially-Stressed Intestinal Epithelium

In both chemically and genetically-induced models of colitis in *Vil-Cre Hmgb1*$^{fl/fl}$ mice, greater severity of the disease is observed (gross measurements of histological intestinal inflammation and body weight loss) (Zhu et al. 2015). At the cellular level, bacterial stress on the cells induce massive intestinal epithelial cell death, more of which occurs without the intracellular presence of HMGB1. Normal bacterial stress induces cell death (observed in Figure 6). This cell death is aggravated without HMGB1 (Figure 7 and Figure 8), due to its protective role in the cytosol (Zhu et al. 2015).

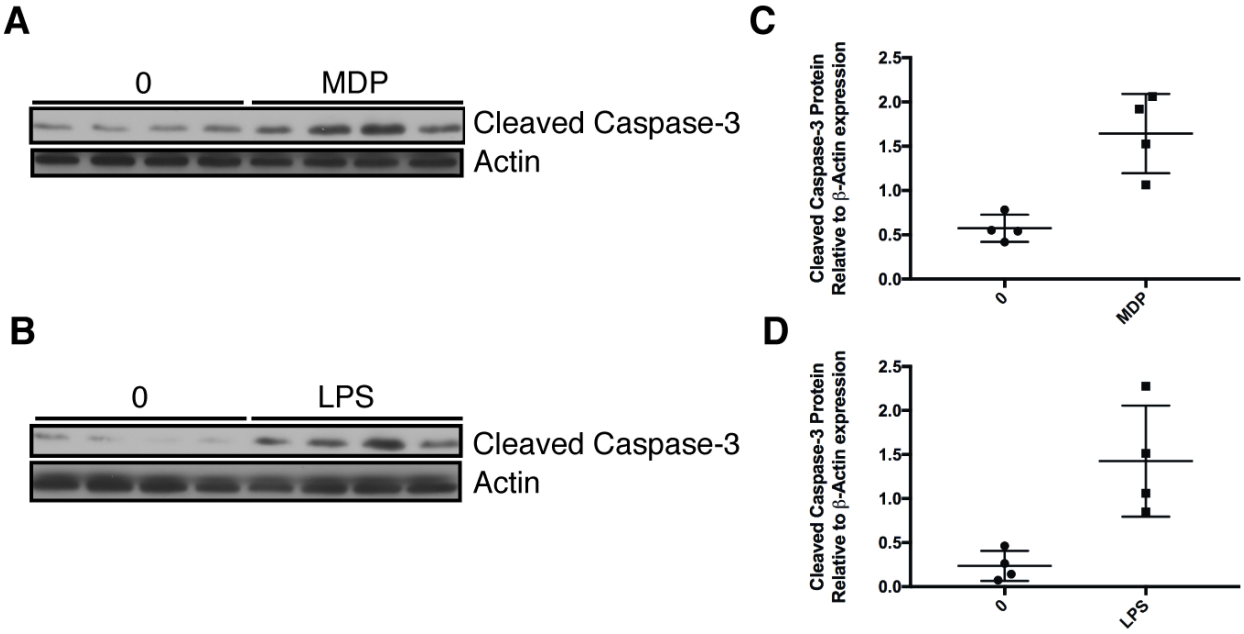

Figure 7: Bacterial Stimuli Induce Cell Death.
A) MDP treatment of four lines of enteroids induces cleaved caspase-3, marker of cell death, as shown by Western blot. B) LPS treatment of four lines of enteroids also induces cell death, C) densitometry of blot and subsequent ratio paired t-test shows greater cleaved caspase-3 from MDP treatment, p-value = 0.0177, D) densitometry of blot and subsequent ratio paired t-test shows greater cleaved caspase-3 from LPS treatment, p-value = 0.0371.

The cell death assay of a western of cleaved caspase-3 was conducted on the protein isolated from the LPS and CpG DNA dose response experiments. The

significance of the dependence on HMGB1 is most apparent at 10 micrograms/ml of LPS, in which the t-test reached a significance of p-value = 0.029. Figure 8A demonstrates that the higher doses of LPS exhibit a greater effect on cell death, as expected and known from the literature (Guo et al. 2013; Guo et al. 2015). For CpG DNA, the two-way ANOVA taking into account both genotype and dose demonstrated that the dose was significant, with a p-value of 0.0019 (Figure 8B). Thus, both LPS and CpG DNA demonstrated a dose-dependence of effect on HMGB1 expression and intestinal cell death.

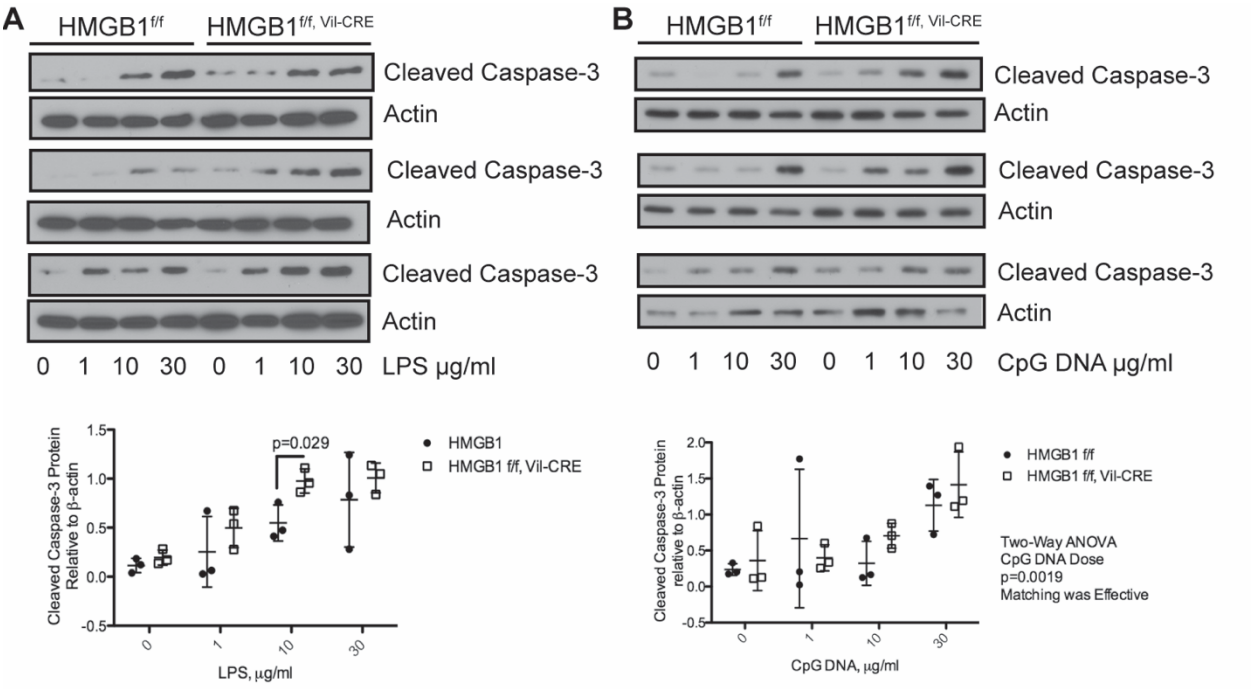

Figure 8: LPS and CpG DNA induce Apoptosis in a Dose-Dependent manner in Enteroids.

A) Cleaved Caspase-3 expression via western blot and quantification of western blot bands from three pairs of HMGB1$^{f/f}$ or HMGB1 $^{f/f, Vil\text{-}CRE}$ enteroid lines treated with doses of LPS (0, 1, 10 and 30 micrograms per milliliter). B) Cleaved Caspase-3 expression via western blot and quantification of western blot bands from three pairs of HMGB1$^{f/f}$ or HMGB1 $^{f/f, Vil\text{-}CRE}$ HMGB1 enteroid lines treated with doses of CpG ODN (0, 1, 10 and 30 micrograms per milliliter).

Further experiments utilized MDP and LPS since these represent the majority of bacteria, representing both gram-negative and gram-positive bacteria. To verify the effects of MDP and LPS, four different lines of enteroids, from independent mouse intestine derivations, were treated with the components and subsequently assayed for HMGB1 protein and the western blots were analyzed by densitometry (Figure 9).

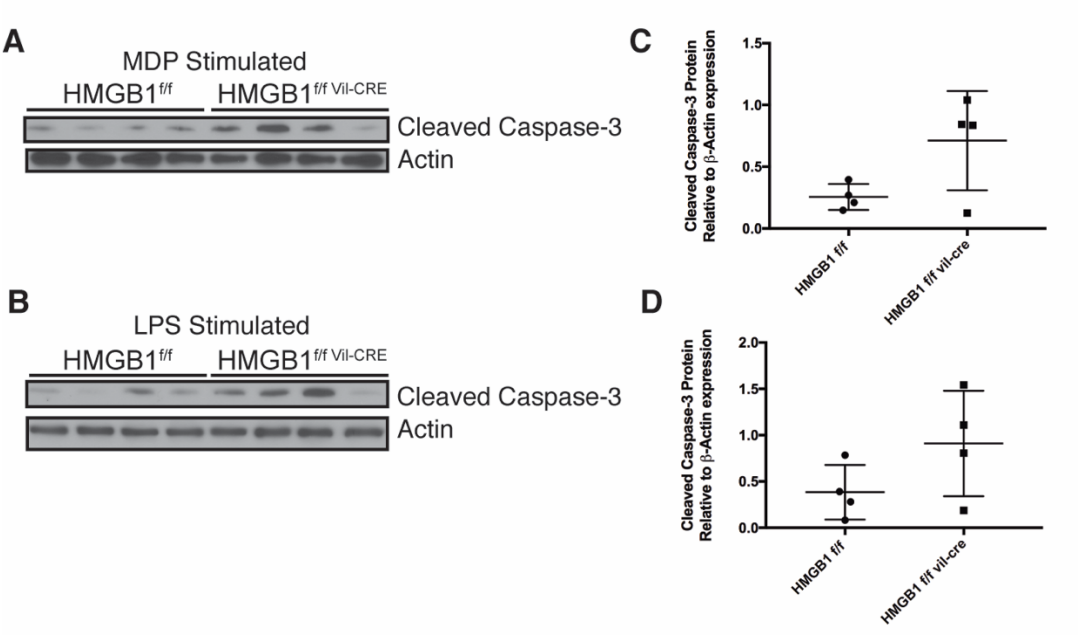

Figure 9: HMGB1 Protects Against Bacterially-Induced Cell Death.
A) HMGB1 dependence of MDP-stimulated cell death, B) HMGB1 dependence of LPS-stimulated cell death in enteroids, C) and D) quantification of A and B respectively.

For further experiments, MDP was utilized as a generic marker of microbial stress to analyze the internal effects on the cell. The next step was to investigate the mechanism of how HMGB1 protects the cell under bacterial stress.

HMGB1 Preserves Cellular Energy Production under Bacterial Stress

In the absence of HMGB1, the intestinal epithelial cells are susceptible to insults because they lack the ability to induce autophagy in the face of external stressors. By protecting Beclin-1 and Atg5 from calpain cleavage, HMGB1 averts the apoptotic fate of the cell (Zhu et al. 2015); yet whether HMGB1 induces autophagy more directly remains unclear, and is even considered to be “dispensable” for autophagy in the liver (Huebener et al. 2014). Since HMGB1 is important in both metabolism and inflammation, the ability to produce and to utilize energy for these pathways is a common mechanism to target. The production of energy generically is marked by ATP levels. While many pathways are involved in ATP production and cellular survival, Akt, or protein kinase B, not only is at the center of these pathways but also downstream of bacterial stress (Wiles et al. 2008). This connection between bacterial induction of the cell’s metabolic and inflammatory pathways likely is compromised in the absence of HMGB1.

In the next set of experiments of treating enteroids with MDP, ATP production and Akt activity were measured. While healthy control cells from the enteroids responded to the bacterial stress by producing ATP, the HMGB1-deficient cells were unable to produce ATP in the face of that stress (Figure 10A). Since HMGB1 dampens calpain activity in its protective function (Zhu et al. 2015), we used the calpain inhibitor, calpeptin, to test the dependence of ATP production in this system. Calpain activity is necessary for the healthy cells to be able to respond and produce these energy stores of ATP. In an investigation of the cell signaling pathways involved in energy production, phosphorylation of Akt was assayed via Western blot over the course of four hours of treatment with MDP. The HMGB1-deficient enteroids were unable to promote Akt

activity to the same level as the healthy controls during this time course (Figure 10B), suggesting that this pathway is compromised under bacterial stress.

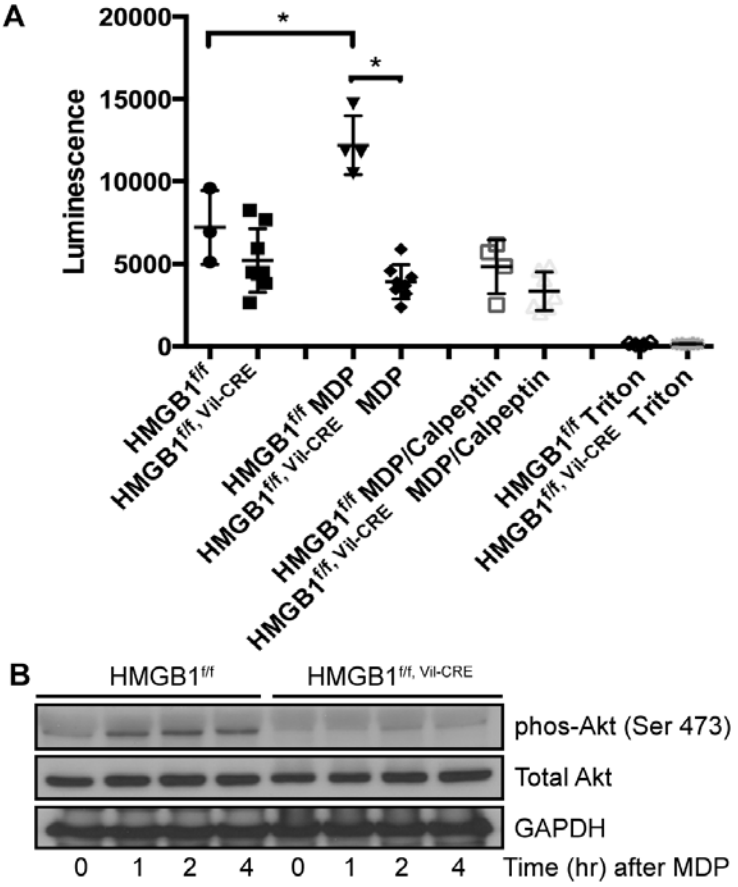

Figure 10: HMGB1-mediated cytoprotection permits cellular energy production during MDP-induced stress.
Enteroids treated with 10 mcg/ml L-18 MDP for the indicated times. A) ATP production as measured by luminescence for intestinal cells treated for 8 hours. B) Immunoblotting for Akt activity over a time course treatment.

Lower levels of HMGB1 in the intestine of inflammatory bowel disease thus represents a severe inability to harness defensive mechanisms against bacterial insults in the heavily bacterially-populated environment of the intestine. As has been observed in patients with active colitis (Zhu et al. 2015), this lower level of HMGB1 in the intestine likely contributes to the inability to survive additional insults, contributing to the

sustained and aggravated cell death that promotes more red, swelling, ulcerated mucosal epithelium observed by physicians. To promote the therapeutic endpoint of mucosal healing for these patients (Neurath and Travis 2012), then, an intervention which promotes cellular energy under microbial stress is needed to provide these diseased intestinal cells with the energy to defend against the insults and sustain cellular survival.

High-Throughput Screening of Approved Drugs for HMGB1-Independent Cellular Energy Production Under Bacterial Stress

The next step in this line of inquiry took advantage of a high-throughput screening (HTS) method to identify approved drug targets that could promote energy production in HMGB1-deficient cells under bacterial stress. This HTS method (depicted in Figure 11) screened the Prestwick Chemical Library (Appendix 2), which includes 1280 small molecules which are 100% approved by the U.S. Food and Drug Administration (FDA), European Medicines Agency (EMA) and other agencies for bioavailability and safety for use in humans.

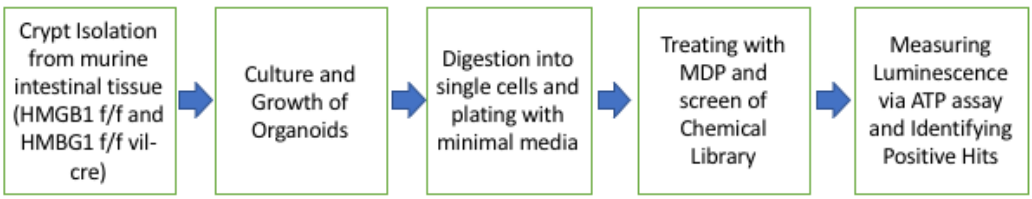

Figure 11: Model of high throughput cellular screening strategy to identify drugs that improve IEC survival in the absence of HMGB1 during microbial stress.

The method assessed whether any of these drugs were able to rescue HMGB1-deficient intestinal epithelial cells under bacterial stress. The raw data from the eight plates is included in Appendices 3-6. Products that generated ATP at more than 25% over the ATP counts from MDP-treated controls, in both replicates of the treatment plate, were the following types: microbial metabolites, drug classes: antimicrobial, chemotherapeutic, statin, or unclassified (see Table 1).

Chemical Name	ATP production Fold Increase	Therapeutic Group
Doxorubicin hydrochloride	2.5	Antibacterial, Immunosuppressor, Antineoplastic
Ribostamycin sulfate salt	2.4	Antibacterial
Chloropyramine hydrochloride	2.3	Antihistaminic
Daunorubicin hydrochloride	2.2	Antineoplastic
Quinacrine dihydrochloride dehydrate	2.0	Antimalarial
Pentamidine isethionate	1.9	Antiparasitic
Adenosine 5'-monophosphate monohydrate	1.9	Antiarrhythmic
Benzylpenicillin sodium	1.9	Antibacterial
Rifabutin	1.8	Antibacterial
Dacarbazine	1.6	Antineoplastic, Antimetabolic
Mitoxantrone dihydrochloride	1.6	Antineoplastic/Anticancer
Calycanthine	1.5	Calcium channel blocker (Mechanism)
Maprotiline hydrochloride	1.5	Antidepressant
Sisomicin sulfate	1.5	Antibacterial
Nadide (nicotinamide adenine dinucleotide)	1.5	Coenzyme nicotinamide adenine dinucleotide (compound)
Ungerine nitrate	1.5	Sedative
Lovastatin	1.4	Antihypercholesterolemic
Nilutamide	1.4	Antineoplastic
Rolitetracycline	1.3	Antibacterial

Table 1: Drugs identified as potential therapies to improve HMGB1-deficient IEC survival during microbial stress.

The next step was to verify that a drug could indeed rescue these intestinal cells from death in the face of bacterial stress. While some IBD therapies already use chemotherapeutic or immunosuppressant approaches, an alternative IBD therapy may be offered by the statin drug class. In fact, statins have been demonstrated to be

protective in mouse models of colitis (Maheshwari et al. 2015; Abe et al. 2012; Lee et al. 2007). Furthermore, human studies show an association of reduced steroid use in IBD patients who are on statins (Crockett et al. 2012), atorvastatin reduced inflammatory markers in Crohn's Disease (CD) patients (Grip et al. 2008; Grip et al. 2009), and there is an ongoing clinical trial using pravastatin in CD patients (NCT00599625). The promise of statin therapy led us to consider the one statin on the list (Table 1).

Lovastatin Treatment of HMGB1-Deficient Cells Under Bacterial Stress

Lovastatin, currently used to prevent cholesterol formation, already has known metabolic effects as an HMG CoA Reductase Inhibitor (Tobert 2003) but has not been tested in a bacterially-stressed intestinal epithelial cell model. While under certain circumstances lovastatin has been shown to lead to intestinal cell death (Agarwal et al. 2002), treatment of Lovastatin in other cell lines has induced cell survival mechanisms. For example, lovastatin regulated the AKT-signaling pathway (Mizushima and Yoshimori 2007), and induced Akt Kinase activation in osteoblasts (Ghosh-Choudhury et al. 2007), while at the same time, appeared to suppress Akt activation in endothelial cells (Prasad et al. 2005). Given that previous work demonstrated that HMGB1 was protective in a murine model of colitis (Zhu et al. 2015), it is also relevant that statins appear to be protective in murine models of colitis (Maheshwari et al. 2015; Abe et al. 2012; Lee et al. 2007) and thus might aid in the HMGB1-deficient condition. We sought to determine whether lovastatin would protect microbially-stressed HMGB1-deficient cells from higher levels of apoptosis.

The effectiveness of the intervention would most readily be apparent by preventing cell death. Thus, active caspase-3 was measured in lovastatin-treated MDP-stressed HMGB1-deficient cells. As seen in Figure 12A, lovastatin was able to mitigate some cell death induced by MDP. To further determine if lovastatin resolved the previously-identified pathway that was compromised in these bacterially-stressed HMGB1-deficient cells, p-AKT blots were performed. These blots (Figure 12) demonstrated that, in fact, lovastatin aided in repairing the compromised pathway of AKT activation in these HMGB1-deficient cells after two hours of treatment.

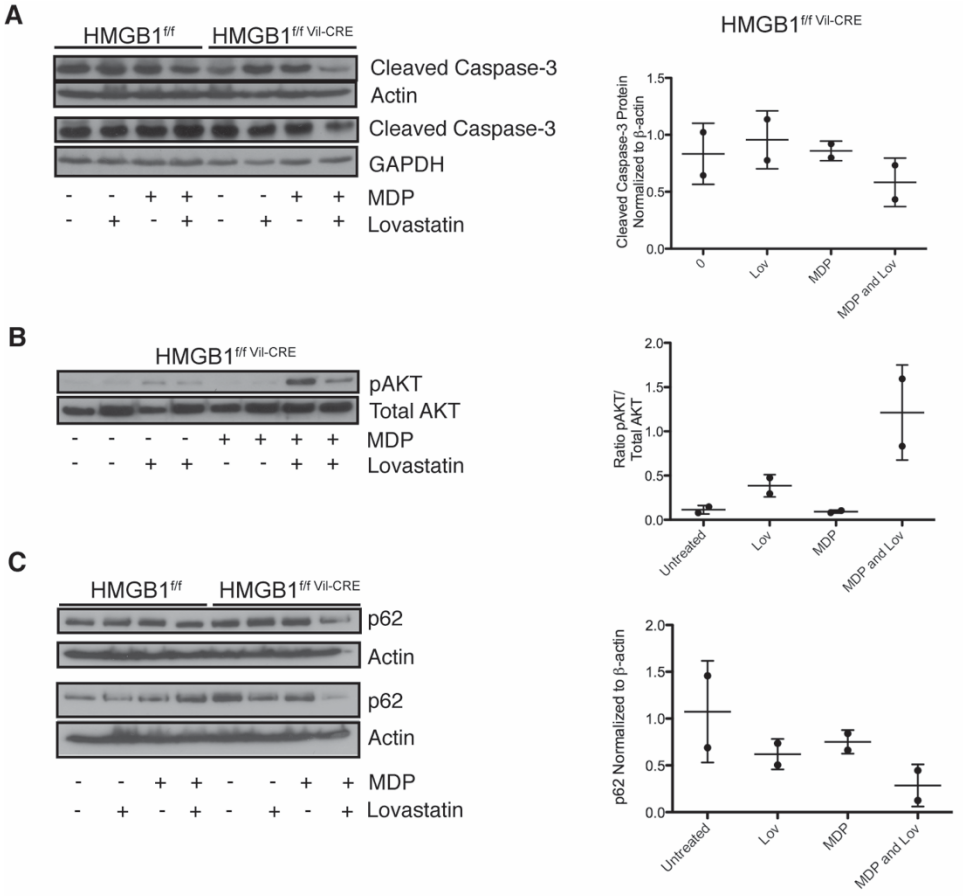

Figure 12: Lovastatin protects HMGB1-deficient cells during microbe-induced stress and rescues Akt phosphorylation with autophagy induction.
Western blot and quantification (HMGB1-deficient cells only) of protein from enteroids treated with or without MDP or Lovastatin: A) Caspase-3 B) Phospho-Akt and C) p62.

Since increased levels of HMGB1 in response to stress are associated with autophagy, and AKT activity is implicated in inhibiting autophagy (Wang et al. *Science* 2012), we sought to understand the ultimate result of these opposing regulators on autophagic proteins. Autophagy, a recycling process of the cell in which it "self-digests" internal components like organelles, cycles through aggregates labeled by the protein p62 (also called Sequestosome-1) such that higher levels of p62 signal that autophagy is inhibited (Bjørkøy et al. 2005). Autophagy appeared to be increased due to the lower levels of accumulated p62 in the MDP and lovastatin combined condition compared to the MDP treated alone condition (Figure 12C). Therefore, this blot suggests that autophagy is aided by the lovastatin treatment when HMGB1-deficient cells experience bacterial stress.

Chapter 4: Discussion

Summary

While HMGB1's extracellular role has received the majority of the literature's attention, this project pursued a mechanistic basis for the protective intracellular role of HMGB1 in colitis susceptibility, and how to rescue the HMGB1-deficient intestinal cells with a drug therapy. This work advances the investigation into cytosolic HMGB1's activity in the intestinal cells and opens new questions regarding its regulatory behavior in the cell's energy production. Beyond the field of knowledge around the protein HMGB1, this work utilizes a model that could be beneficial in other diseases and for personalized therapy by a high-throughput screen of drugs with a simple read-out of treated primary cells from organoids.

This work has further described the intracellular role of HMGB1 and the drug lovastatin as follows and in Figure 13: Bacterial components increase HMGB1 expression in a dose-dependent manner, HMGB1 allows AKT activity to preserve cellular energy availability (ATP) in intestinal epithelial cells under microbial stress as demonstrated in these experiments in the mouse enteroid model, proper levels of AKT activity in the intestinal epithelial cell may be modulated by bacteria and HMGB1 and the drug lovastatin which ultimately affect cell survival or proliferation through a combination of inflammatory/metabolic pathways., and in bacterially-stressed HMGB1-deficient IEC, lovastatin treatment mitigates cell death by promoting AKT activity. In addition to the drug lovastatin, this screen identified eighteen other drugs which present opportunities for further research and evaluation for IBD therapies.

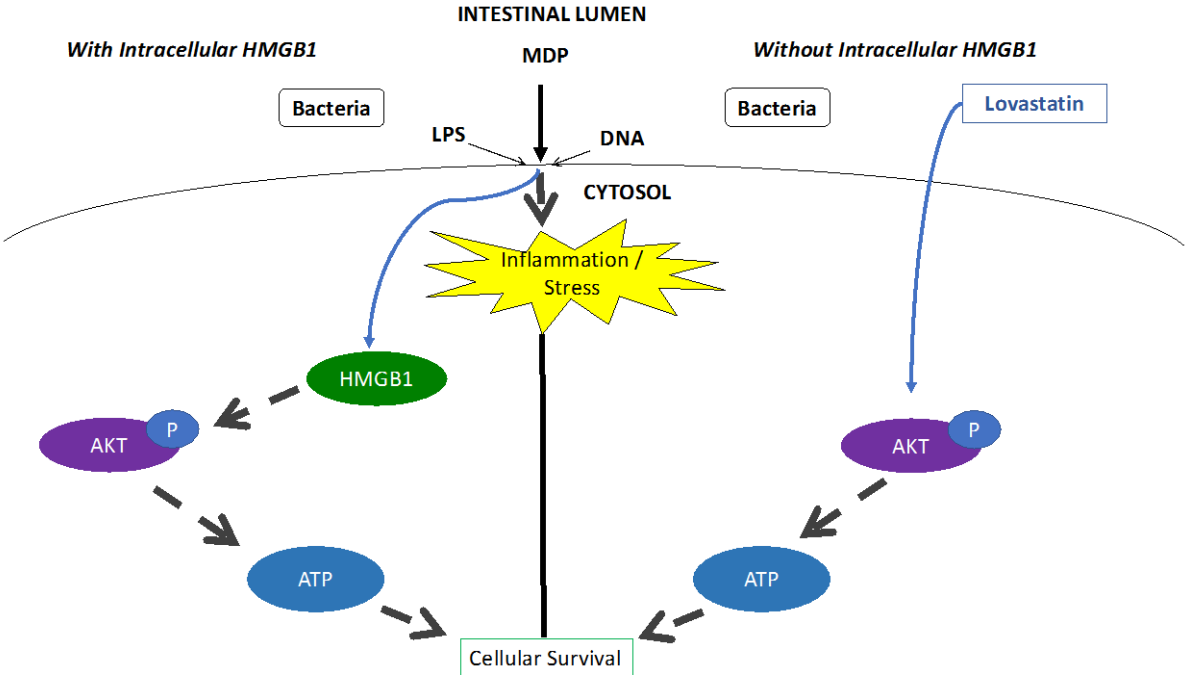

Figure 13. Summary of Molecular Signaling Observed in the Presence and Absence of HMGB1 to Promote Cellular Survival.
Bacterial stress leads to increased cell death in the absence of intracellular HMGB1. This study demonstrated that the presence of HMGB1 promotes cellular survival by allowing the phosphoryltation of AKT and preserving cellular energy (ATP production) during bacterial stress of IEC. In the absence of intracellular HMGB1, lovastatin prevents the susceptible cells from dying by rescuing AKT activity for the cell's energy production needs.

Limitations and Expansion of this Study

Aspects of the design and methodology of this study limit the conclusions and implications from the results. First of all, these studies were performed in enteroids, derivatives of the small intestine, from which it could be argued that this study applies more to Crohn's Disease. Further tests could be performed in colonoids to be more applicable to UC. Initial tests in colonoids of MDP treatment and HMGB1 expression were not completely consistent and colonoid growth was more technically challenging and unreliable than enteroid growth which resulted in the focus of enteroids for this

study. Additionally, the tissue was from mice; human tissue would be more relevant. Human biopsies could be sourced for future experiments. Secondly, the configuration of this organoid model is an inverted intestinal epithelium in spheroid structures. A more physiological relevant system would utilize a monolayer and deliver the treatment to the apical side. Many cells are required to seed such a monolayer and this system was not available at the time of this study.

Third, it was also difficult to determine how to translate physiologically relevant concentrations of bacterial factors from a monolayer or *in vivo* system to the organoid *in vitro* system here. As an example, we observed in the western blot that 0 and 1 microgram per ml of LPS had similar effects (Figure 8). This could be due to the fact that physiologically relevant concentrations of LPS are 0 to 1 ng/ml and has been measured at 1.8 micrograms per ml in the rat lumen; additionally, LPS may not cause cell death but increase selective intestinal permeability (Guo et al. 2013). Thus, while the bacterial factor concentrations utilized in these experiments likely are many fold higher than the *in vivo* concentrations, the larger concentration may have been necessary to induce a response as there needed to be more available to penetrate the matrigel and be accessible to the cells; or conversely, may have induced more cell death than would be expected because much fewer cells were present than in a monolayer system. It is difficult to compare concentrations since the concentration of the enteroids, which is not a single cell layer, is probably not the same as the number of intestinal cells that were exposed to that concentration; therefore, a monolayer experiment would need to be done to be more physiologically relevant.

Fourth, the stability of the organoid model has not been characterized. This study limited the passage number of the enteroids used because passage number 21 enteroids exhibited more cell death and did not respond similarly to lower passage enteroids. We have not assessed whether the enteroids changed in genetic expression over this long course of passaging. For example, a recent report demonstrated that a different media condition altered the characteristics of jejunum-derived organoids (Han et al. 2017). While we did not change our media conditions during the culturing process, freezing and thawing the cells in recovery cell media, which should theoretically not alter the cells, could have had some type of effect. At the same time, cellular differences and chromosomal abnormalities were not observed in long-term culture of human and mouse intestinal organoids using a method similar to that used in this study (Sato et al. 2011). While we did not observe gross differences under the microscope and do not anticipate that genomic changes occurred, we have not performed rigorous genetic expression profiles to assess for transcriptome differences across low to high passage number cells.

In the evaluation of the nuclear and cytosolic distribution of HMGB1 in the cell (see Figure 6), we noted that this organoid system likely already induces severe stress on the cells and thus they may behave differently than the *in vivo* system. The media contains a high level of glucose and high levels of growth factors which provide a heavy stimulation of the cells to proliferate; the model could be developed with less glucose and lower growth factors to balance the growth needs with the cell's ability to mimic the *in vivo* system. Relevant readouts of the derivative organism's intestine such as transcriptomic profiles or at minimum, mRNA and protein of the target proteins and

known mechanistic effects could be used for the optimization of this *in vitro* system while varying the contents of the media. Other studies demonstrate, for example, that glucose conditions and diabetic models exhibit higher levels of HMGB1 in the cytosol (Chen et al. 2015; Kim et al. 2016), which may be occurring in our model and also suggests another disease to evaluate for HMGB1's intracellular role using the pipeline suggested here (discussed in The Future of IBD Therapy section) and potentially with other cell type-derived organoids.

Despite its limitations, the model still utilizes primary intestinal epithelial cells and not transformed cells and is thus a "near-physiological model system" (Fatehullah et al. 2016). Further studies within this model could evaluate not only various doses of bacterial components, but also other types of bacterial components and bacterial metabolites (short chain fatty acids and deoxycholic acid (DCA) for example). Particularly relevant would be to examine various IBD-associated bacteria types and components as contrasted to "healthy/control"-associated microbial communities and their metabolites. Generally, there is less diversity of microbes in the IBD patient's intestine, and bacterial populations on the mucosal biopsies of UC and CD patients have less *Clostridium* IXa and IV groups, *Bacteroides*, bifidobacteria and more sulphate-reducing bacteria, *Proteobacteria*, *Actinobacteria, and Enterobacteriaceae* and *Escherichia coli* (Fava and Danese 2011; Huttenhower et al. 2014). Lower butyrate is observed, likely due to less *Clostridium* in the intestine (Huttenhower et al. 2014). With antibiotic treatment common, dysbiosis may be exacerbated as the overall levels of bacteria decrease. Heat-treatment or sterile-filtration of these bacteria yielding bacterial components would provide an IBD-associated or control-associated milieu for treatment

in the organoid model used in this project. For a hypothesis, all the bacterial components might stimulate HMGB1 expression to a certain extent, and would exhibit a dose-dependent relationship; the effects would potentially differ with respect to the cell death assay as more inflammation may overwhelm the cells treated with more IBD-associated milieu. Since butyrate is a major energy source for the cells, I would expect that metabolite to increase HMGB1 and protect cell health from increased cell death.

Further characterization within this system could evaluate a time course of assessing cell death and autophagy, mRNA and protein evaluation, Enzyme linked-immunosorbent assays (ELISAs), and other techniques like immunohistochemistry of cut sections. These studies could also be done simply with *ex vivo* biopsies, if enough tissue was obtained, with shorter time courses prior to cell death to avoid the extensive time and cost of growing out organoids or to mitigate the challenges of the fixation and sectioning of small organoids.

The study was somewhat limited by use of the Prestwick Chemical Library, which contains a predetermined set of compounds in predetermined forms at preset concentrations. It is possible that other compounds at different concentrations might have increased ATP levels and been considered a hit using our criterion. It would be possible to pipette more of each compound into this system as is to use higher concentrations; however, higher concentrations risk toxicity while lower concentrations risk missing possible hits. Additionally, other forms that were more bioavailable or different metabolites or byproducts of the compounds may have been able to increase ATP levels as well. It is also interesting to note that this same chemical library has been used to try to repurpose drugs for IBD using a zebrafish neutrophil migration assay (Hall

et al. 2014); however, their approach still sought drugs that suppressed the immune system (anti-inflammatory activities) rather than promoted energy production and cell survival (the focus of this thesis). Overall, the ability to screen 1280 compounds rapidly provided a huge advantage in this study to narrowing the number of compounds that could potentially rescue the compromised system in this project.

After evaluating and screening bacterial factors and components *in vitro*, taking the bacterial factors into the *in vivo* system under colitic conditions would challenge the effect of bacterial factors and the screened drugs in relation to the complexity of the whole organism in the disease model. Feeding bacterial components or metabolites to germ-free mice would provide an alternative model to isolate the effects of specific bacterial factors to assess if HMGB1 expression would be induced in the intestine and also to examine the incidence of increased colitis on a larger level using DSS- or $IL10^{-/-}$ -inducement of colitis and the presence or absence of HMGB1 in the IEC. At the same time, it is important to note that these “germ-free” mice are raised in a sterile environment which means that there are not live bacteria present, but does not exclude the possibility that bacterial components may still be entering through water or food for example.

As previously mentioned, others have already conducted *in vivo* experiments in colitic mice with MDP (Watanabe et al. 2008), LPS (Makimura et al. 2007; Im et al. 2011); yet *B. subtilis*-derived LTA, and this particular type of CpG DNA as well as the FLA used in this study could also be tested in colitic mouse models. While other statins have been demonstrated to be protective in mouse models of colitis, lovastatin can be tested in the specific case of colitis-susceptible mouse model *Vil-Cre* $Hmgb1^{fl/fl}$ mice with

colitis induced by DSS or IL10$^{-/-}$ alongside these bacterial factor-association studies. Thus, the synergy of the bacterial factor and drug within the HMGB1-deficient, colitic-susceptible intestine *in vivo* could be evaluated on the whole organism level.

The *in vivo* studies would also address another limitation of this study, which is that it focuses on intestinal epithelial cells, the innate immune response, and does not address the adaptive immune response. IBD has been associated with dysregulated immune responses, which suggests that, even if the IEC may function properly, there could exist other defects independent of the HMGB1-dependent/associated mechanism such as microbial defense and antimicrobial activity, reactive oxygen species generation, T helper 17 and regulatory T cell activity (Huttenhower et al. 2014). These pathways will likely need to be addressed in conjunction, as HMGB1 has been shown to affect microbial defense and reactive oxygen species (Tang et al. 2011; Yu et al. 2015) and T cells (Wild et al. 2012; Lotfi et al. 2016; Cheng et al. 2017) but these references researched extracellular HMGB1. Extracellular HMGB1 was not measured in the study written in this thesis because the focus was on the role of the less well characterized intracellular HMGB1. It is possible that extracellular HMGB1 would still be relevant in the *Vil-Cre Hmgb1$^{fl/fl}$* mouse as it would come from macrophages or other cells and trigger downstream effects which could be altered due to the lack of HMGB1 in the intestine and would thus be relevant to the current model. Extracellular HMGB1 could be measured by western blots or ELISAs of HMGB1 in the media, as well as recombinant HMGB1 treatment (provided that the HMGB1 is not bacterially-derived, in which it may be carrying bacterial components) of the relevant immune cell populations *in vitro* to assess how HMGB1, or rather, the absence of HMGB1, in *Vil-Cre Hmgb1$^{fl/fl}$*

mice affects the adaptive immune system's response. Additionally, the bacterial components could be triggering the IEC to signal to the immune cells (e.g., flagellin in Sierro et al. 2001) and since these mechanisms would be co-occurring, the challenge of the drug therapy in the colitic *Vil-Cre Hmgb1*$^{fl/fl}$ mice would better assess how well lovastatin or another drug hit could perform beyond recovering the ability to preserve cellular energy in IEC. The drug's effect on other cell types and the drug absorption, delivery, metabolism, and excretion pattern would be better assessed in the whole organism in which intracellular HMGB1 regulation of energy metabolism and extracellular HMGB1 impact on antimicrobial defense and immune signaling may be further examined.

The interaction with genetics was not investigated here, but is relevant to pursue in other studies (models examining the role of NOD2 for example) such as using genetic knockout models of mice that also combine the HMGB1 colitis-susceptible model. A Crohn's disease patient may be unable to respond appropriately to bacteria in general due to loss-of-function mutations in NOD2 (Abreu et al. 2002), which not only drives excessive inflammation, but could, according to this paper's findings, lead to lower HMGB1 levels, less autophagy/cellular survival, and therefore more cell death and aggravated symptoms. While HMGB1 has never been implicated in a GWAS for CD or any other disease, these other genetic factors that increase susceptibility to IBD could be concomitantly present or exacerbate colitis when HMGB1 is also deficient, and thus drugs or therapies identified in the screening would need to be evaluated for effects that would also benefit those with the genetic predisposition for IBD. Thus, studies in a model of a whole organism would better demonstrate the ultimate impact on colitis

through the integration of the innate immune and metabolic responses and consideration of the impact on genetically-susceptible models with respect to these various treatments.

The Mechanism of AKT Activation

HMGB1's role in allowing phosphorylation of AKT signaling under bacterial stress could be further interrogated in future studies, and lovastatin's role in assisting in AKT activation in the absence of HMGB1 needs further characterization. While we noted that AKT activity was increased in a short time course, we acknowledge that the negative feedback of the PI3K/AKT pathway on NOD2-mediated NF-kb (Zhao et al. 2008) competes with the bacterial stimulation of NOD2, and thus varying the doses of MDP and the duration of treatment may result in more complicated scenarios which would require deeper analysis of cell health parameters and counting cell populations to understand the overall effect of a particular treatment condition. As our studies demonstrated, HMGB1, and lovastatin in the absence of HMGB1, may help activate p-AKT when the cell is bacterially-stressed while MDP otherwise reduces p-AKT via NOD2 (Tamrakar et al. 2010). Similar to the MDP and Lovastatin effect, MDP has also been shown to synergistically induce phosphorylation of AKT with TLR4 agonist Monophosphoryl Lipid A (MPLA) (Tukhvatulin et al. 2016). Thus, the amount of bacteria will be influential in the drug dose-sensitivity as the complex signaling mechanisms encounter multiple stimuli.

Further *in vitro* studies examining duration of treatment, cell type, co-culturing of cell types, and various doses of multiple agents would better characterize the various players in these metabolic and inflammatory pathways. Because AKT is central to so many other pathways, further investigation into other pathways involving AKT activation and cellular survival might pinpoint a molecule that HMGB1 directly inhibits or activates through binding. To find which molecule HMGB1 may bind within the AKT pathway, future investigation into this mechanism could utilize bioinformatics to determine possible binding sites of HMGB1 and interactions with other molecules involved in AKT pathways if an HMGB1 structure becomes available (Guo and Wang 2012; Pang et al. 2012). Additionally, since phosphorylation of AKT is also important for the other site, Threonine, the phosphorylation of Threonine could be examined as well. pAKT inhibitors could be utilized in the *in vitro* system to assess whether molecules downstream of AKT are affected. Due to the complexity of the feedback regulation, PI3K inhibitor LY294002 may be useful in suppressing the PI3K/Akt pathway in addition to mitigating the feedback of pAKT activation (Chen et al. 2013). It may not be that pAKT itself is the rate limiting aspect; a molecule upstream of AKT activation could be affected. Lovastatin has been shown to suppress N-methyl-D-Aspartate (NMDA)-induced Glycogen synthase kinase 3 beta (GSK3β) activation in neuronal cells (Ma et al. 2009), and while GSK3β is downstream from AKT, and this study demonstrated that lovastatin activated pAKT, it is worth evaluating this and other downstream molecules (Manning and Cantley 2007).

Since HMGB1 protects calpain-client proteins from cleavage under bacterial stress (Zhu et al. 2015), the calpain activity which is relevant in the AKT signaling pathways

may be overactive and thus any drug treatment such as lovastatin, that modulates calpain activity (Ma et al. 2009) may prevent the calpain activity from being overactive in the absence of HMGB1's shielding effect on its targets. Additionally, since bacteria may trigger the inflammatory pathways through calpain as well, the bacteria in the dysbiotic milieu of the IBD patient or IBD model should be evaluated with respect to this mechanism and its effect on IEC survival. For example, *H. pylori* activated calpain in a monolayer of human gastric epithelial cells and thus other bacteria and bacterial types could also be active in the small and large intestine in inducing calpain activity (O'Connor et al. 2011). HMGB1 deficiency under bacterial stress also disadvantages the cell through its inability to perform autophagy, because autophagy assists in clearing bacteria from inside the cells and thus is a necessary protective cellular function in CD (Lassen et al. 2014). Thus, repeating the studies in this thesis for evaluation of calpain activity and more autophagy characterization as well as analyzing calpain activity and autophagy proteins over a time course in further studies of the various other drug hits in this study would elucidate the role of calpain and characterize the autophagy process in more detail and improve understanding of these mechanisms.

To ensure that HMGB1 can rescue these cells from microbe-induced death, studies that add back HMGB1 to the HMGB1-deficient cells can test cytosolic HMGB1 sufficiency for cellular survival under bacterial stress. These experiments can utilize viral transduction and overexpression of the protein HMGB1 in the HMGB1 deficient cells. We would expect to observe less cell death as measured by cleaved caspase-3 Western blots in MDP-stressed HMGB1-deficient cells that were virally transduced with the HMGB1 versus the MDP-stressed control virally-transduced HMGB1-deficient cells.

That experiment would further confirm that HMGB1 in the cytosol can sufficiently protect the cells from death. However, the HMGB1 may also be present at higher levels in the media and affect the experiment as an exogenous activator of NF-kB signaling through binding to RAGE and downstream signaling (Lotze and Tracey 2005), though this has not yet been demonstrated in primary epithelial cells. Therefore, these experiments would require careful analysis of HMGB1 protein in each compartment (nuclear, cytosolic, and extracellular) in addition to cell viability, calpain activity, and caspase-3 to determine if HMGB1, and in which compartment HMGB1 is acting, is associated with less cell death. Finally, different forms of HMGB1, which can occur due to posttranslational modifications like acetylation, have different roles, and these roles require more research (Hu et al. 2015) as to their function for the localization and activity of HMGB1. Thus, the over expression of HMGB1 would also need to be monitored for which form is present and if it is the same as the endogenous cytosolic form that appears to be contributing to cellular survival.

The Future of IBD Therapy

While immunosuppressive drugs remain the mainstay of IBD therapy, novel use of repurposing drugs and targeting the microbiome may provide more options for patients to achieve mucosal healing, even as their current drug treatments become ineffective. This work demonstrated with molecular support that statins may be viable, while at the same time, a statin is currently undergoing a clinical trial. Other drug hits in this study could be further pursued as possible treatments for IBD, and further HTS of various

other compounds, such as nutraceuticals, dietary components, probiotic and prebiotic components, may identify other types of therapy.

Interestingly, there were other statins, besides lovastatin, included in the Prestwick Chemical Library that did not increase ATP production in our screen. Fluvastatin and simvastatin failed to satisfy our screening criteria for increased ATP production; atorvastatin was not included in the screen. There are a number of differences among the different drugs that make up the statin drug class. Lovastatin and simvastatin are type 1 statins that have substituted decalin-ring structures (Pahan 2006). Lovastatin is a natural product derived from fungi whereas simvastatin is a semi-synthetic derivative of lovastatin (Manzoni and Rollini 2002). Atorvastatin is a type 2 statin that is fully synthetic and has larger side groups linked to the HMG-like moiety (Istvan 2003). Therefore, related drugs may not be functionally equivalent. All of the statins were developed for use in treatment of hypercholesterolemia and are still primarily prescribed for this use. For this reason, they have been almost exclusively studied for their activity as HMG-CoA (3-hydroxy-3-methylglutaryl-CoA) reductase inhibitors. Statins are metabolized in the liver to activate their anti-HMG-CoA (3-hydroxy-3-methylglutaryl-CoA) reductase activity. However, we used unmetabolized lovastatin in our studies. This is an important distinction since IEC would be expected to be primarily exposed to the unmetabolized form of the drug. It also supports an alternative function for lovastatin in IEC during microbe-induced stress (Liao and Laufs 2005).

The Prestwick Chemical library also included drugs that are currently used for inflammatory bowel disease or utilize mechanisms (antibacterial or anti-inflammatory for example) that have been targeted as an approach for IBD. IBD-indicated drugs

including sulfasalazine (anti-inflamatory), mesalamine (anti-inflammatory), prednisone (corticosteroid), hydrocortisone (corticosteroid), azathioprine (immunosuppressive), methotrexate (chemotherapy), metronidazole (antibiotic) and ciproflaxcin (antibiotic) did not elevate ATP levels in our HTS above control levels. These drugs were not targeted to promote cellular viability, which is the approach we took to identify new drugs. Thus, it is an interesting finding that these drugs do not promote cellular energy production, or rescue the HMGB1-deficient IEC stressed by bacteria. These drugs did not depress the luminescence counts to the level of background, or "no cell" control values; thus, I do not expect that they generated massive cell death. Perhaps a treatment that affects the immune cells (as these drugs are already available and in use) could be combined with a therapy, that behaves like lovastatin did in our system, to promote intestinal epithelial cell viability to maintain the intestinal epithelial barrier.

While lovastatin was selected for validation in our study, the HTS identified 18 other compounds that elevated ATP levels over 1.25 fold in MDP-challenged, HMGB1-deficient IEC. These other drugs could be evaluated for their effects on IEC viability, as potentially beneficial for mucosal healing of IBD damage. The various compounds differ in chemical structures and published mechanistic action. Some are commonly used while others are not in clinical use. There were other drugs besides lovastatin that have been tested and shown to mitigate rodent colitis, though these studies did not provide IEC-specific data. For example, quinacrine is an anti-malarial drug that improved overall health by suppressing inflammation in mice with chemically-induced colitis (Chumanevich et al. 2016). The anti-protozoal pentamidine mitigated colitis in an animal model (Esposito et al. 2012). Maprotiline, a norepinephrine reuptake inhibitor,

ameliorated acetic-acid induced colitis in rats (Minaiyan et al. 2014). The maprotiline study also suggested that utilization of anti-depressants in IBD would be beneficial because of the higher incidence of depression in IBD patients than in the control population. While there were other anti-depressants on the list of drug hits, searches did not reveal that any of these anti-depressants have been tested in colitic models or for their effects on intestinal cells. While the specific tetracycline compound rolitetracycline showed up on our list, a generic tetracycline was used as part of combination therapy (Kato et al. 2014), a follow-up study from the same lab that previously published on these combination antibiotic therapies (Uehara et al. 2010; Ohkusa et al. 2010). Patients were treated as part of the "clinical routine"; thus, their medications may have been fluctuating and different patient-to-patient, confounding the effects of antibiotic treatment in the study. Nonetheless, the authors reported that clinical activity indices concluded that in 63.3% of steroid refractory and 73.4% of steroid dependent patients a clinical response was observed within 2 weeks (Kato et al. 2014) which may sound promising but requires further research to such as assessment of mucosal healing and long-term remission. While a metaanalysis of antibiotic use in IBD concludes that antibiotics benefit both CD and UC, it may be that patients on antibiotics also received more aggressive drug management of their disease and thus were more likely to improve based on clinicians' supervision and expertise (Wang et al. *Exp Ther Med* 2012). Antibiotic usage is complicated by the known effect on the microbiome that indirectly affects the IEC whereas the antibiotic effect directly on IEC is less well studied.

Other antibacterial agents in Table 1 have been listed in a patent for using antibiotics with GI cleansers to help IBD patients (US 2009/0324736 A1), allowing for the possibility of these drugs to be used in IBD in the future. Thus, those agents may be worth investigating in rodent colitis models as well. On the other hand, these same drugs that may be considered for treatment may initiate or aggravate the disease. Stomach and intestine pain arises as the side effect with the highest average importance for patients on anti-depressants (Wouters et al. 2014). Antibiotic usage preceded five case reports of acute, transient colitis (Toffler et al. 1978). Specifically, this last report included penicillin, a derivative of which, benzylpenicillin, was identified in the HTS. Penicillin has also been implicated in antibiotic-associated diarrhea in pediatric patients (Kuehn et al. 2015). This may reflect dose-dependencies or effects on the gastrointestinal microbial community that were not recapitulated in our screen due to the lack of the luminal environment in our in vitro system. It shows the importance of further investigation of potential drugs of interest in physiologic animal models.

One potential confounder of the antibiotic approach, as has been observed in this study, is that the presence of bacteria promotes HMGB1 expression. Thus, the lower levels of HMGB1 in patients with active colitis may be due to lower levels of bacteria or bacterial factors that promote HMGB1 expression, and antibiotic treatment could exacerbate the loss of the protective function of intracellular HMGB1 expression by removing the bacterial factors that promote it. On the other hand, if the patients resemble those in the previous study (Zhu et al. 2015), in which the mRNA was high and showed a bottleneck in HMGB1 expression, the HMGB1 protein is lower due to the patient's inability to manufacture HMGB1 protein. Independent of HMGB1, the presence

of bacteria and its components could also affect other signaling pathways through AKT activity and thus impact cellular survival. Thus, the bacterial modulators of antibiotics, prebiotics, and probiotics would need to be carefully controlled in assessing future therapies, and these combinations (at minimum, antibiotic usage, which is already widespread in the clinic for IBD) with the drug, such as lovastatin or the other drugs on the list, could be tested in the colitic *Vil-Cre Hmgb1*$^{fl/fl}$ mouse model prior to advancing the therapy for clinical research. Besides antibiotics, using statins in addition to existing standard IBD therapies (which was already evaluated as associated with less steroid use in the Crockett et al. 2012 publication) could be evaluated at the molecular and mechanistic level to fine tune dose recommendations and later in patients to establish dose recommendations as a statin/steroid or statin/biologic combination therapy in the future.

Since diet also alters the microbiome, and thus, changes in diet will likely affect how the patient responds to the therapeutic treatment, diet, at least with respect to specific fat content, may need to be controlled or evaluated (Devkota et al. 2012) in these future studies. Not only that, but diet may be a critical aspect of the therapeutic regimen as discussed in the introduction of this thesis due to the high occurrence of malnutrition. Dietary components themselves may have direct effects on the IEC and promote cell viability. Our HTS did evaluate vitamins, amino acids and other nutrients that did not increase ATP levels 1.25 fold over those in MDP-stressed HMGB1-deficient IEC. These included the following: Kynurenine, 3-hydroxy (R,S), Panthenol (D), Pantothenic acid calcium salt monohydrate, Pyridoxine hydrochloride, Cyanocobalamin, Ascorbic acid, Aspartic acid, N-acetyl (R,S), Folic acid, retinoic acid, Cholecalciferol and calciferol,

vitamin K2, Capsaicin, Folinic acid calcium salt, N-Acetyl-L-leucine, and Niacin. It is important to note that these were not tested in healthy wild-type cells as these compounds may promote ATP production in an HMGB1-dependent manner; alternatively, they may be effective in promoting cellular energy production at other concentrations or not under bacterial stress. It is interesting to observe that while niacin (nicotinic acid and nicotinamide/vitamin B3) did not elevate ATP levels above our set threshold, nadide (nicotinamide adenine dinucleotide, the active form of nicotinamide) did, highlighting the importance of the proper form of the compound for efficacy. Other compounds to consider evaluating could be bacterial metabolites (for HMGB1-independent mechanisms) such as butyrate, which could also be a proxy for supplements or the FODMAP diet and investigate a probiotic mechanism of affecting cell viability and therefore mucosal healing.

In this study, we showed that lovastatin rescued Akt signaling in HMGB1 deficient IEC exposed to MDP. There is conflicting data from the literature about how lovastatin influences Akt signaling. In the majority of studies using transformed cells, lovastatin inhibited Akt activation (Klawitter et al. 2010; Ma et al. 2012; Zhao et al. 2010). In the majority of studies using primary cells, lovastatin promoted Akt phosphorylation (Prasad et al. 2005; Ghosh-Choudhury et al. 2007). None of these studies examined Akt phosphorylation in the context of microbial stress. The apparent inconsistencies in Akt phosphorylation appear to be dependent upon cell type with very different effects in primary and transformed cells. Our studies using primary IEC showed that lovastatin-induced Akt phosphorylation was also dependent on the cellular context since lovastatin alone failed to induce Akt phosphorylation while lovastatin treatment during MDP

induced cellular stress did induce Akt phosphorylation. Additionally, we showed that lovastatin rescued autophagy in MDP treated, HMGB1-deficient IEC. Lovastatin alone did not induce autophagy in our model, but autophagy proceeded in the presence of lovastatin when induced by MDP in HMGB1-deficient cells. This is particularly interesting in light of our published data showing that HMGB1 is required for protection of beclin-1 and Atg5 from calpain mediated conversion from pro-autophagic to pro-apoptotic proteins during MDP induced autophagy. Akt signaling and autophagy are intertwined at the level of autophagy activation through PI3K/Akt/mTor signaling pathways. From our data it is not clear whether lovastatin rescues Akt phosphorylation directly or indirectly through preservation of autophagy. One clue may be that lovastatin has also been reported to modulate calpain activity (Ma et al. 2009), which would be expected to rescue autophagic activity in MDP stimulated HMGB1 deficient cells. Calpain inhibition has been associated with major side effects through inhibition of cellular repair pathways, but calpain modulation may allow those pathways to progress, without the detrimental effects of calpain activation on cell processes (Ji et al. 2016).

Utilizing the PI3K/AKT/PTEN pathway is already under investigation for Crohn's disease therapy (Tokuhira et al. 2015). Dietary supplements (such as fish oil, curcumin, linoleic acid, and wormwood) and drugs modulate AKT activity as well as regulate autophagy, and several have been investigated in the treatment of IBD in experimental as well as clinical trials (Tokuhira et al. 2015). Lovastatin's activity, while shown to be similar to HMGB1's role in our studies as increasing AKT activity, has also been shown to inhibit calpain (Ma et al. 2009), another approach to preventing cell death by stopping excessive inflammation. Our study demonstrated that calpain was necessary for

HMGB1-sufficient cells to respond with increased energy production under bacterial stress which points to the need to investigate quantitatively for activity in future work. At the same time, we recognize that lovastatin's ability to promote ATP production was not directly investigated; it would be interesting to examine lovastatin at various doses, without HMGB1, and its interaction with bacterial factors to identify how significant its activity is relative to other influences. Since lovastatin itself is a fungal secondary metabolite, derived from *Aspergillus terreus* (Endo et al. 1992), it could be interacting with the microbial and fungal populations in the intestine, considering the whole organism. Interestingly, simvastatin, which was shown to be beneficial in mouse models of colitis, did not induce ATP production (though it was one of the compounds tested from the Prestwick library) and apparently has been shown to phosphorylate AKT (Kureishi et al. 2000). Thus, the structure and properties of the lovastatin compound, from which simvastatin is derived, must be important to its role in our experimental system. These studies were limited to the reductionist model of mini-guts in culture, whereas the more complex system of the whole host would be susceptible to many other modulators of AKT activity, autophagy, and other mechanisms relevant to intestinal epithelial cell health.

Predictions may be made as to whether some of the other drugs on the list in Table 1 would also increase phosphorylation of AKT. Since Nilutamide blocks the androgen receptor, I would expect it to activate AKT signaling, because inhibition of the androgen receptor decreases the AKT phosphatase Pleckstrin Homology domain Leucine-rich repeat Protein Phosphatase (PHLPP) and thus activates AKT (Carver et al. 2011). At the same time, the reference also demonstrates that the PI3K and AR pathway provide

reciprocal negative feedback. PI3K is activated when AR is inhibited, and PI3K then can activate AKT. Ungerine nitrate may generate nitric oxide which may also activate AKT (Mejia-Garcia et al. 2013). The antidepressant Maprotiline has been demonstrated to be involved in autophagic cell death (Cloonan and Williams 2011), so it is unclear whether it would promote cell death or cell survival pathways, but it is worth evaluating since lower AKT activity is associated with depression (Karege et al. 2007) and Akt regulates autophagy (Wang et al. *Science* 2012).

Current clinical trials for IBD include probiotics and statins, reflecting the contemporary significance of the findings in this work. The ability to promote autophagy, to modulate AKT activity and to prevent cellular death through microbial and statin therapies shows promise for aiding in mucosal healing for patients with inflammatory bowel disease. This type of work can be continued as part of a pipeline for development of drugs designed to promote mucosal healing in IBD (see Figure 14). Beginning with cellular models and high throughput approaches, drug hits can be moved into *in vivo* models and then patient trials. Additionally, cellular models could even be generated from patient tissue (although active lesions usually have too much cell death to provide any substantial amount of healthy cells to grow and be treated with this approach). The concept is to identify new IBD therapeutics that promote mucosal healing with minimal side effects.

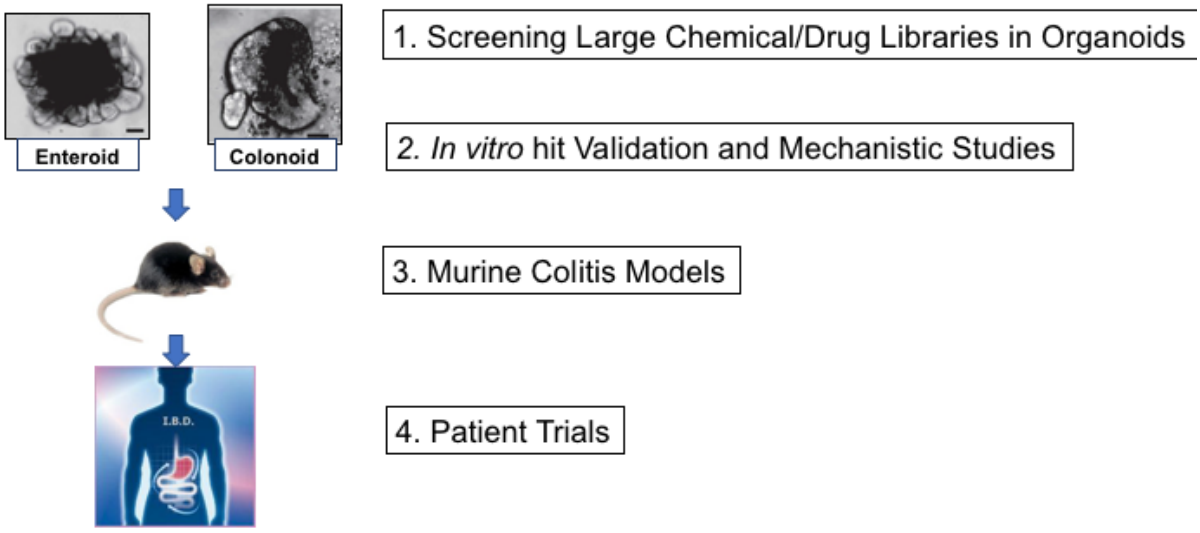

Figure 14. Drug development pipeline for identification of novel IBD therapies designed to promote mucosal healing.

Overall, our data from high throughput screening to *in vitro* validation using the drug hit lovastatin show that this approach is applicable to patient disease, can be used to rapidly screen existing drugs or novel compounds for use in IBD, and serves as a platform for *in vitro* mechanistic investigation and validation of candidate drugs. We are not proposing lovastatin as a radical new therapy for IBD, but rather suggesting that this and other drugs identified in our screen may be safe, inexpensive, adjuncts to current therapies. More importantly for the purposes of developing novel IBD therapies, our studies of lovastatin have validated our approach to identify high value drug targets that can now be further investigated using more expensive and time-consuming *in vivo* models. Not only can we now investigate additional drugs identified in the screen using our *in vitro* model system of HMGB1 deficient IEC, but we can also perform *in vivo* studies using the mice conditionally deficient in IEC HMGB1 from which the cells were originally derived. We can additionally use these same techniques to perform screens

with other drugs libraries or libraries of compounds in development for use as drugs. Finally, this approach has the potential to be developed for personalized medicine using organoids derived from patients who are refractory to therapy. This type of approach is rapid, relatively inexpensive and doesn't require *a priori* knowledge of which pathways to target. This means that it also has the potential to identify new mechanistic targets for directed drug development. The hope is that this type of drug discovery pipeline from cellular models to clinical studies in patients can lead to major advances in therapy and ultimately, cures for IBD and other diseases related to microbe-induced cellular responses.

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Appendix 1

HTS Plate Map of Treatment and Genotype

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	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
A			-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	
B			-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	
C			-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	
D			-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	
E			-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	
F			-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	
G			-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	
H			-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	
I			-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	+/+	
J			-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	+/+	
K			-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	+/+	
L			-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	+/+	
M			-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	+/+	
N			-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	+/+	
O			-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	+/+	
P			-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	+/+	

no cells in first and last columns

untreated

(4 plates whole library x 2 replicate plates each= 8 total plates)

MDP + compound

MDP alone

-/- = HMGB1 fl/fl vil-CRE

+/+ = HMGB1 fl/fl or WT

Appendix 2
Prestwick Chemical Compound Plate Layout
(384 well plates)

1

	1	2	3	4	5	6
A			Azaguanine-8	Metronidazole	Allantoin	Cotinine (-)
B			Isoniazid	Mexiletine hydrochloride	Pentylenetetrazole	Flavoxate hydrochloride
C			Meticrane	Khellin	Benzonatate	Zimelidine dihydrochloride monohydrate
D			Tranexamic acid	Chlorothiazide	Etofylline	Diphenidol hydrochloride
E			Sulfaphenazole	R(-) Apomorphine hydrochloride hemihydrate	Panthenol (D)	Amoxapine
F			Ampyrone	Ethisterone	Levamisole hydrochloride	Triprolidine hydrochloride
G			Chloramphenicol	Naloxone hydrochloride	Epirizole	Metolazone
H			Midodrine hydrochloride	Vincamine	Thalidomide	Indomethacin
I			Procaine hydrochloride	Bromocryptine mesylate	Moxisylyte hydrochoride	Metanephrine hydrochloride DL
J			Ranitidine hydrochloride	Fludrocortisone acetate	Tiratricol, 3,3',5-triiodothyroacetic acid	Fenoterol hydrobromide
K			Trichlorfon	Glipizide	Carbamazepine	Loxapine succinate
L			Piroxicam	Dantrolene sodium salt	Pyrantel tartrate	Trazodone hydrochloride
M			Morantel tartrate	Verapamyl hydrochloride	Homatropine hydrobromide (R,S)	Dipyridamole
N			Norfloxacin	Lisinopril	Antimycin A	Lincomycin hydrochloride
O			Todralazine hydrochloride	Erythromycin	Imipramine hydrochloride	Oleandomycin phosphate
P			Etodolac	Ifenprodil tartrate	Scopolamin-N-oxide hydrobromide	Flunarizine dihydrochloride

2

	1	2	3	4	5	6
A			Streptomycin sulfate	Testosterone propionate	Alfuzosin hydrochloride	Arecoline hydrobromide
B			Serotonin hydrochloride	Tubocurarine chloride pentahydrate (+)	Cefotiam hydrochloride	Dihydroergocristine mesylate
C			Tremorine dihydrochloride	Androsterone	Practolol	Anisomycin
D			Metixene hydrochloride	Tetracaïne hydrochloride	Nitrofural	Mometasone furoate
E			Furosemide	Suloctidil	Methapyrilene hydrochloride	Carcinine
F			Vigabatrin	Hydrastine hydrochloride	Biperiden hydrochloride	Lobelanidine hydrochloride
G			Chlorthalidone	Coralyne chloride hydrate	Dobutamine hydrochloride	Corticosterone
H			Fluoxetine hydrochloride	Laudanosine (R,S)	Iohexol	Ajmalicine hydrochloride
I			Bambuterol hydrochloride	Estradiol-17 beta	Betamethasone	Fusaric acid
J			Glycocholic acid	Scoulerine	Thiostrepton	Ajmaline
K			Ketotifen fumarate	Kynurenine, 3-hydroxy (R,S)	Debrisoquin sulfate	Lactobionic acid
L			Pirenperone	Harmalol hydrochloride dihydrate	Isoquinoline, 6,7-dimethoxy-1-methyl-1,2,3,4-tetrahydro, hydrochloride	Harmol hydrochloride monohydrate
M			Lidoflazine	Tropisetron HCl	Betaxolol hydrochloride	Cefixime
N			Ciclopirox ethanolamine	Methoxy-6-harmalan	Probenecid	Stachydrine hydrochloride
O			Terbutaline hemisulfate	Strophantine octahydrate	Ketanserin tartrate hydrate	Pantothenic acid calcium salt monohydrate
P			Hexetidine	Thiamine hydrochloride	Selegiline hydrochloride	Dipivefrin hydrochloride

Appendix 2
Prestwick Chemical Compound Plate Layout
(384 well plates)

3

	1	2	3	4	5	6
A			Sulmazole	Althiazide	Epicatechin-(-)	Isopyrin hydrochloride
B			Carbinoxamine maleate salt	Niacin	Methazolamide	Bemegride
C			Glimepiride	Sulfaquinoxaline sodium salt	Picrotoxinin	Streptozotocin
D			Bergenin monohydrate	3-Acetylcoumarin	Cromolyn disodium salt	Esculin Hydrate
E			Vitexin	Nadide	Gelsemine	Sulfamethizole
F			Azlocillin sodium salt	Hymecromone	Clidinium bromide	Caffeic acid
G			Dydrogesterone	Terazosin hydrochloride	(d,l)-Tetrahydroberberine	Phenazopyridine hydrochloride
H			Butacaine	(+)-Isoproterenol (+)-bitartrate salt	Cefoxitin sodium salt	Monobenzone
I			Austricine hydrate	Butamben	beta- Belladonnine dichloroethylate	Sulfapyridine
J			Securinine	Nizatidine	Trimeprazine tartrate	Thioperamide maleate
K			Fillalbin	Alclometasone dipropionate	Citalopram Hydrobromide	Leflunomide
L			Paroxetine Hydrochloride	Propofol	Doxycycline hyclate	S(-)Eticlopride hydrochloride
M			Trihexyphenidyl-D,L Hydrochloride	Clobetasol propionate	Succinylsulfathiazole	Podophyllotoxin
N			Gabapentin	Pentetic acid	Raloxifene hydrochloride	Bretylium tosylate
O			Sulfabenzamide	(R) -Naproxen sodium salt	Benzocaine	Propidium iodide
P			Iopamidol	Crotamiton	Iopromide	Propranolol hydrochloride

4

	1	2	3	4	5	6
A			Denatonium benzoate	Etomidate	Scopoletin	Tridihexethyl chloride
B						
C			Remoxipride Hydrochloride	Moricizine hydrochloride	THIP Hydrochloride	Iopanoic acid
D						
E			Nitrocaramiphen hydrochloride	Phensuximide	Rilmenidine hemifumarate	Ioxaglic acid
F						
G			Gliquidone	Imidurea	Pizotifen malate	Lansoprazole
H						
I			Alfadolone acetate	(S)-propranolol hydrochloride	Alfaxalone	(-)-Eseroline fumarate salt
J						
K			Flucloxacillin sodium	Spaglumic acid	Trapidil	(-)-Adenosine 3',5'-cyclic monophosphate
L						
M			Isradipine	Netilmicin sulfate	Tiletamine hydrochloride	Loracarbef
N						
O			Halofantrine hydrochloride	Mecamylamine hydrochloride	Articaine hydrochloride	Procarbazine hydrochloride
P						

Appendix 2

Prestwick Chemical Compound Plate Layout

(384 well plates)

1

	7	8	9	10
A	Acetazolamide	Edrophonium chloride	Metformin hydrochloride	Moroxidine hydrochloride
B	Chlorzoxazone	Bufexamac	Ornidazole	Glutethimide, para-amino
C	Hydroflumethiazide	Azacyclonol	Sulfacetamide sodic hydrate	Azathioprine
D	Tranylcypromine hydrochloride	Norethindrone	Alverine citrate salt	Nortriptyline hydrochloride
E	Sulfadiazine	Cyproheptadine hydrochloride	Norethynodrel	Famotidine
F	Pargyline hydrochloride	Doxepin hydrochloride	Methocarbamol	Dyclonine hydrochloride
G	Diprophylline	Ciprofloxacin hydrochloride	Triamterene	Ampicillin trihydrate
H	Oxolinic acid	Cortisone	Nimesulide	Prednisolone
I	Betazole hydrochloride	Dehydrocholic acid	Isoxicam	Hesperetin
J	Flufenamic acid	Homochlorcyclizine dihydrochloride	Flumequine	Diethylcarbamazine citrate
K	Triflupromazine hydrochloride	Hydroxyzine dihydrochloride	Mefenamic acid	Diltiazem hydrochloride
L	Fenspiride hydrochloride	Glafenine hydrochloride	Gemfibrozil	Pimethixene maleate
M	Nifedipine	Chlorhexidine	Chlorpromazine hydrochloride	Loperamide hydrochloride
N	Xylometazoline hydrochloride	Telenzepine dihydrochloride	Oxymetazoline hydrochloride	Econazole nitrate
O	Sulindac	Midecamycin	Amitryptiline hydrochloride	Josamycin
P	Hyoscyamine (L)	Trifluoperazine dihydrochloride	Chlorphensin carbamate	Enalapril maleate

2

	7	8	9	10
A	Chlorpropamide	Thyroxine (L)	Phenylpropanolamine hydrochloride	Tocopherol (R,S)
B	Azathymine, 6	Noscapine	Benperidol	Syrosingopine
C	Zidovudine, AZT	Carbarsone	Sulfisoxazole	Apigenin
D	Omeprazole	Tomatidine	Propylthiouracil	Dacarbazine
E	Desipramine hydrochloride	Carisoprodol	Clorgyline hydrochloride	Cephalosporanic acid, 7-amino
F	Cetirizine dihydrochloride	Papaverine hydrochloride	Etifenin	Yohimbine hydrochloride
G	Enoxacin	Cyanocobalamin	Clopamide	Cefadroxil
H	Norcyclobenzaprine	Trigonelline	Pyrazinamide	Diclofenac sodium
I	Colchicine	Gabazine	Metergoline	Ginkgolide A
J	Methionine sulfoximine (L)	Monocrotaline	Tiabendazole	Piperlongumine
K	Amethopterin (R,S)	Lumicolchicine gamma	Methylergometrine maleate	Lysergol
L	Phenacetin	Harmine hydrochloride	Atovaquone	Ellipticine
M	Nicardipine hydrochloride	Metrizamide	Probucol	Muramic acid, N-acetyl
N	Betahistine mesylate	Pyridoxine hydrochloride	Tobramycin	Cytisine (-)
O	Hemicholinium bromide	Cefotetan	Kanamycin A sulfate	Piperine
P	Pentamidine isethionate	Thiorphan	Tolazamide	Riboflavine

Appendix 2
Prestwick Chemical Compound Plate Layout
(384 well plates)

3

	7	8	9	10
A	Flunisolide	Phenethicillin potassium salt	N-Acetyl-DL-homocysteine Thiolactone	Sulfamethoxypyridazine
B	Pyrithyldione	Digoxigenin	Spectinomycin dihydrochloride	Meglumine
C	Mepenzolate bromide	Metoprolol-(+,-) (+)-tartrate salt	Benfotiamine	Flumethasone
D	Bucladesine sodium salt	Felbinac	Cefsulodin sodium salt	Butylparaben
E	Solasodine	Medrysone	Delcorine	Flunixin meglumine
F	Sulfamonomethoxine	Diloxanide furoate	Benzthiazide	Metyrapone
G	Deltaline	Demeclocycline hydrochloride	Graveoline	Fenoprofen calcium salt dihydrate
H	Ifosfamide	2-Aminobenzenesulfonamide	Novobiocin sodium salt	Estrone
I	Pempidine tartrate	Meclofenoxate hydrochloride	Heliotrine	Furaltadone hydrochloride
J	Nafcillin sodium salt monohydrate	Xamoterol hemifumarate	Procyclidine hydrochloride	Rolipram
K	Promazine hydrochloride	Norgestrel-(-)-D	Sulfamerazine	Fluocinonide
L	Liothyronine	Primidone	Roxithromycin	Flucytosine
M	Famprofazone	Clofibric acid	Bromopride	Bendroflumethiazide
N	Etidronic acid, disodium salt	Pralidoxime chloride	Methylhydantoin-5-(D)	Phenoxybenzamine hydrochloride
O	Dipyrone	Cloperastine hydrochloride	Isosorbide dinitrate	Eucatropine hydrochloride
P	Theophylline monohydrate	(R)-(+)-Atenolol	Theobromine	Tyloxapol

4

100

	7	8	9	10
A	Enilconazole	Penbutolol sulfate	Methacycline hydrochloride	Prednicarbate
B				
C	Pirlindole mesylate	Pivmecillinam hydrochloride	Pronethalol hydrochloride	Levopropoxyphene napsylate
D				
E	Dimaprit dihydrochloride	Naftifine hydrochloride	Reserpinic acid hydrochloride	Meprylcaine hydrochloride
F				
G	Ribavirin	Bethanechol chloride	Cyclopenthiazide	Cyproterone acetate
H				
I	Azapropazone	Condelphine	Meptazinol hydrochloride	Leucomisine
J				
K	Deptropine citrate	(-)-Quinpirole hydrochloride	Sertraline	Sulfadoxine
L				
M	Isometheptene mucate	Quinic acid	Nifurtimox	Natamycin
N				
O	Nomegestrol acetate	Viomycin sulfate	Pancuronium bromide	Saquinavir mesylate
P				

Appendix 2

Prestwick Chemical Compound Plate Layout

(384 well plates)

1

	11	12	13	14
A	Atracurium besylate	Baclofen (R,S)	Isoflupredone acetate	Acyclovir
B	Ethosuximide	Dropropizine (R,S)	Mafenide hydrochloride	Pinacidil
C	Heptaminol hydrochloride	Lynestrenol	Sulfathiazole	Guanabenz acetate
D	Aceclofenac	Niflumic acid	Iproniazide phosphate	Isotretinoin
E	Thiamphenicol	Danazol	Cimetidine	Nicorandil
F	Aztreonam	Dimenhydrinate	Cloxacillin sodium salt	Disopyramide
G	Dapsone	Haloperidol	Troleandomycin	Naltrexone hydrochloride dihydrate
H	Hydrastinine hydrochloride	Fenofibrate	Pentoxifylline	Bumetanide
I	Naproxen	Perphenazine	Naphazoline hydrochloride	Mefloquine hydrochloride
J	Tolfenamic acid	Chenodiol	Meclofenamic acid sodium salt monohydrate	Perhexiline maleate
K	Acetohexamide	Methotrexate	Sulpiride	Astemizole
L	Mefexamide hydrochloride	Pergolide mesylate	Tiapride hydrochloride	Acemetacin
M	Diphenhydramine hydrochloride	Chlortetracycline hydrochloride	Minaprine dihydrochloride	Tamoxifen citrate
N	Nifenazone	Bupivacaine hydrochloride	Griseofulvin	Clemastine fumarate
O	Adiphenine hydrochloride	Paclitaxel	Dibucaine	Ivermectin
P	Metampicillin sodium salt	Minocycline hydrochloride	Dilazep dihydrochloride	Glibenclamide

2

101

	11	12	13	14
A	Ascorbic acid	Pepstatin A	Methyldopa (L,-)	SR-95639A
B	Cefaclor	Atropine sulfate monohydrate	Colistin sulfate	Eserine sulfate, physostigmine sulfate
C	Zaprinast	Aspartic acid, N-acetyl (R,S)	Chlormezanone	Bacampicillin hydrochloride
D	Terconazole	Ipratropium bromide	Tiaprofenic acid	Acetopromazine maleate salt
E	Clenbuterol hydrochloride	Chicago sky blue 6B	Maprotiline hydrochloride	Buflomedil hydrochloride
F	Metaproterenol sulfate, orciprenaline sulfate	Lobeline alpha (-) hydrochoride	Sisomicin sulfate	Berberine chloride
G	Hycanthone	Cyclosporin A	Adenosine 5'-monophosphate monohydrate	Digitoxigenin
H	Trimethadione	Calycanthine	Lovastatin	Convolamine hydrochloride
I	Brinzolamide	Cyclobenzaprine hydrochloride	Ambroxol hydrochloride	Carteolol hydrochloride
J	Rifampicin	Hydrocotarnine hydrobromide	Ethionamide	(-)-Cinchonidine
K	Methiothepin maleate	Mebhydroline 1,5-naphtalenedisulfonate	Clofazimine	Meclocycline sulfosalicylate
L	Methoxamine hydrochloride	Chrysene-1,4-quinone	(S)-(-)-Atenolol	Demecarium bromide
M	Mitoxantrone dihydrochloride	Myricetin	GBR 12909 dihydrochloride	Naringenine
N	Tetramisole hydrochloride	Pseudopelletierine hydrochloride	Pregnenolone	Racecadotril
O	Amikacin hydrate	Brompheniramine maleate	Etoposide	Primaquine diphosphate
P	Nifuroxazide	Hydroquinine hydrobromide hydrate	Mycophenolic acid	Epivincamine

Appendix 2
Prestwick Chemical Compound Plate Layout
(384 well plates)

3

	11	12	13	14
A	Flurandrenolide	Deferoxamine mesylate	Helveticoside	Mephentermine hemisulfate
B	Piromidic acid	Cantharidin	Trimipramine maleate salt	Clioquinol
C	Halcinonide	Flecainide acetate	Lanatoside C	Cefazolin sodium salt
D	Fosfosal	Aminohippuric acid	Suprofen	N-Acetyl-L-leucine
E	Evoxine	Spiramycin	(cis-) Nanophine	Glycopyrrolate
F	Trichlormethiazide	Urapidil hydrochloride	Oxalamine citrate salt	Fluspirilen
G	Hippeastrine hydrobromide	Piperacillin sodium salt	Beta-Escin	Diethylstilbestrol
H	Tetrahydroxy-1,4-quinone monohydrate	Lorglumide sodium salt	Indoprofen	Nitrendipine
I	Nitrarine dihydrochloride	Ethoxyquin	Lycorine hydrochloride	Tinidazole
J	Amiprilose hydrochloride	Thonzonium bromide	Ethynylestradiol 3-methyl ether	Idazoxan hydrochloride
K	Acacetin	Sulfamethazine sodium salt	Ethotoin	Guaifenesin
L	Beclomethasone dipropionate	(-)-MK 801 hydrogen maleate	Tolmetin sodium salt dihydrate	Bephenium hydroxynaphthoate
M	Methyl benzethonium chloride	Dicumarol	(1-[(4-Chlorophenyl)phenyl-methyl]-4-methylpiperazine)	Methimazole
N	Simvastatin	Salmeterol	Azacytidine-5	Altretamine
O	Sulfachloropyridazine	Isocarboxazid	Pramoxine hydrochloride	Lithocholic acid
P	Reserpine	Florfenicol	Arcaine sulfate	Megestrol acetate

4

	11	12	13	14
A	Gibberellic acid	Sertaconazole nitrate	Sotalol hydrochloride	Repaglinide
B				
C	Naftopidil dihydrochloride	Piperidolate hydrochloride	Tracazolate hydrochloride	Trifluridine
D				
E	Beta-sistosterol	Milrinone	Proscillaridin A	Methantheline bromide
F				
G	Fluvoxamine maleate	(R)-Propranolol hydrochloride	Cefalonium	Ciprofibrate
H				
I	Apramycin	Dubinidine	Epitiostanol	D-cycloserine
J				
K	Ethamsylate	Cyclopentolate hydrochloride	Moxonidine	Estriol
L				
M	Letrozole	Verteporfin	Arbutin	Meropenem
N				
O	Molindone hydrochloride	Ronidazole	Alcuronium chloride	Dorzolamide hydrochloride
P				

Appendix 2
Prestwick Chemical Compound Plate Layout
(384 well plates)

1

	15	16	17	18
A	Amiloride hydrochloride dihydrate	Diazoxide	Amprolium hydrochloride	Amidopyrine
B	Riluzole hydrochloride	Albendazole	Nitrofurantoin	Clonidine hydrochloride
C	Levodopa	Disulfiram	Idoxuridine	Acetylsalicylsalicylic acid
D	Sulfamethoxazole	Retinoic acid	Mephenesin	Antazoline hydrochloride
E	Doxylamine succinate	Tomatine	Ethambutol dihydrochloride	Nomifensine maleate
F	Nalidixic acid sodium salt hydrate	Clotrimazole	Pentolinium bitartrate	Vinpocetine
G	Pyrimethamine	Chlorpheniramine maleate	Hexamethonium dibromide dihydrate	Nalbuphine hydrochloride
H	Metaraminol bitartrate	Labetalol hydrochloride	Salbutamol	Cinnarizine
I	Ticlopidine hydrochloride	Isoconazole	Dicyclomine hydrochloride	Spironolactone
J	Kawain	Oxybutynin chloride	Trimethoprim	Spiperone
K	Benoxinate hydrochloride	Clindamycin hydrochloride	Oxethazaine	Terfenadine
L	Mebendazole	Benzydamine hydrochloride	Fenbufen	Fipexide hydrochloride
M	Miconazole	Nicergoline	Isoxsuprine hydrochloride	Canrenoic acid potassium salt
N	Clemizole hydrochloride	Oxytetracycline dihydrate	Tropicamide	Pimozide
O	Prednisone	Gallamine triethiodide	Thioridazine hydrochloride	Neomycin sulfate
P	Ofloxacin	Guanethidine sulfate	Lomefloxacin hydrochloride	Quinacrine dihydrochloride dihydrate

2

	15	16	17	18
A	Cefoperazone dihydrate	Adamantamine fumarate	Zoxazolamine	Butoconazole nitrate
B	Daunorubicin hydrochloride	Aconitine	Dosulepin hydrochloride	Rescinnamin
C	Procainamide hydrochloride	Betulinic acid	N6-methyladenosine	Biotin
D	Vancomycin hydrochloride	Rauwolscine hydrochloride	Artemisinin	Corynanthine hydrochloride
E	Thioguanosine	Chlorogenic acid	Chlorprothixene hydrochloride	Cephaeline dihydrochloride heptahydrate
F	Quercetine dihydrate	Chelidonine (+)	Resveratrol	Galanthamine hydrobromide
G	Amoxicillin	Digoxin	Cephalexin monohydrate	Doxorubicin hydrochloride
H	Nystatine	Isocorydine (+)	Budesonide	Xylazine
I	Benfluorex hydrochloride	Hydrocortisone base	Bepridil hydrochloride	Hydroxytacrine maleate (R,S)
J	Tenoxicam	Eburnamonine (-)	Triflusal	Cinchonine
K	Nafronyl oxalate	Meclozine dihydrochloride	Bezafibrate	Melatonin
L	Piracetam	Quipazine dimaleate salt	Phenindione	Sparteine (-)
M	Carbetapentane citrate	Naringin hydrate	Dequalinium dichloride	Neostigmine bromide
N	Molsidomine	Folic acid	Chloroquine diphosphate	Salsolinol hydrobromide
O	Clomiphene citrate (Z,E)	Progesterone	Oxantel pamoate	Felodipine
P	Dirithromycin	Retrorsine	Gliclazide	Conessine

Appendix 2
Prestwick Chemical Compound Plate Layout
(384 well plates)

3

	15	16	17	18
A	Myosmine	Ergocryptine-alpha	Betonicine	Sulfadimethoxine
B	Chloropyramine hydrochloride	Oxybenzone	Furazolidone	Promethazine hydrochloride
C	Benzamil hydrochloride	Atractyloside potassium salt	Suxibuzone	Folinic acid calcium salt
D	Catechin-(+,-) hydrate	Pipemidic acid	Nadolol	Dioxybenzone
E	Foliosidine	Cefamandole sodium salt	Skimmianine	Monensin sodium salt
F	Propantheline bromide	S-(+)-ibuprofen	Lasalocid sodium salt	Ethynodiol diacetate
G	Gossypol	Chlorotrianisene	Ricinine	Ribostamycin sulfate salt
H	Carbenoxolone disodium salt	Flurbiprofen	Iocetamic acid	Nimodipine
I	Karakoline	Guanadrel sulfate	Estropipate	Vidarabine
J	(-) -Levobunolol hydrochloride	Quinapril HCl	Iodixanol	Nilutamide
K	3-alpha-Hydroxy-5-beta-androstan-17-one	Alexidine dihydrochloride	Tetrahydrozoline hydrochloride	Proadifen hydrochloride
L	(+) -Levobunolol hydrochloride	Dehydroisoandosterone 3-acetate	Doxazosin mesylate	Benserazide hydrochloride
M	Diphenylpyraline hydrochloride	Merbromin	Benzethonium chloride	Hexylcaine hydrochloride
N	Paromomycin sulfate	Prazosin hydrochloride	Acetaminophen	Timolol maleate salt
O	Finasteride	Methotrimeprazine maleat salt	Fluorometholone	Dienestrol
P	Scopolamine hydrochloride	Deoxycorticosterone	Ioversol	Urosiol

4

	15	16	17	18
A	6-Hydroxytropinone	Piretanide	Decamethonium bromide	Piperacetazine
B				
C	Zardaverine	Oxprenolol hydrochloride	Memantine Hydrochloride	Ondansetron Hydrochloride
D				
E	Sanguinarine	Ticarcillin sodium	Harpagoside	Thiethylperazine malate
F				
G	Fluticasone propionate	Tropine	Zuclopenthixol hydrochloride	Benzylpenicillin sodium
H				
I	Fursultiamine Hydrochloride	2-Chloropyrazine	Gabexate mesilate	(+,-)-Synephrine
J				
K	Etilefrine hydrochloride	(-)-Isoproterenol hydrochloride	Alprostadil	Kaempferol
L				
M	Tocainide hydrochloride	Ramipril	Benzathine benzylpenicillin	Mephenytoin
N				
O	Zalcitabine	Azaperone	Methyldopate hydrochloride	Cefepime hydrochloride
P				

Appendix 2

Prestwick Chemical Compound Plate Layout

(384 well plates)

1

	19	20	21	22	23	24
A	Hydrochlorothiazide	Ursolic acid	Sulfaguanidine	Pindolol		
B	Hydralazine hydrochloride	Bupropion hydrochloride	Phenelzine sulfate	Alprenolol hydrochloride		
C	Captopril	Mianserine hydrochloride	Minoxidil	Nocodazole		
D	Phenformin hydrochloride	Ethacrynic acid	Flutamide	Praziquantel		
E	Antipyrine	Dizocilpine maleate	Antipyrine, 4-hydroxy	Acenocoumarol		
F	Aminopurine, 6-benzyl	Clomipramine hydrochloride	Tolbutamide	Fendiline hydrochloride		
G	Diflunisal	Picotamide monohydrate	Niclosamide	Triamcinolone		
H	Prilocaine hydrochloride	Methylprednisolone, 6-alpha	Camptothecine (S,+)	Quinidine hydrochloride monohydrate		
I	Amyleine hydrochloride	Pirenzepine dihydrochloride	Lidocaïne hydrochloride	Dexamethasone acetate		
J	Metoclopramide monohydrochloride	Pyrilamine maleate	Fenbendazole	Sulfinpyrazone		
K	Pheniramine maleate	Cefotaxime sodium salt	Tolazoline hydrochloride	Tetracycline hydrochloride		
L	Ketoprofen	Mifepristone	Indapamide	Diperodon hydrochloride		
M	Acebutolol hydrochloride	Thioproperazine dimesylate	Tolnaftate	Dihydroergotamine tartrate		
N	Nefopam hydrochloride	Amodiaquin dihydrochloride dihydrate	Phentolamine hydrochloride	Mebeverine hydrochloride		
O	Diphemanil methylsulfate	Dihydrostreptomycin sulfate	Trimethobenzamide hydrochloride	Gentamicine sulfate		
P	Orphenadrine hydrochloride	Clofilium tosylate	Proglumide	Fluphenazine dihydrochloride		

2

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	19	20	21	22	23	24
A	Tacrine hydrochloride hydrate	Amiodarone hydrochloride	Bisoprolol fumarate	Amphotericin B		
B	Ceftazidime pentahydrate	Dihydroergotoxine mesylate	Iobenguane sulfate	Emetine dihydrochloride		
C	Guanfacine hydrochloride	Bisacodyl	Domperidone	Calciferol		
D	Propafenone hydrochloride	Palmatine chloride	Ethamivan	Trimethylcolchicinic acid		
E	Ritodrine hydrochloride	Cholecalciferol	Clozapine	Cisapride		
F	Bromperidol	Bicuculline (+)	Cyclizine hydrochloride	Yohimbinic acid monohydrate		
G	Dextromethorphan hydrobromide monohydrate	Carbimazole	Droperidol	Epiandrosterone		
H	Imipenem	Seneciphylline	Sulfasalazine	Boldine		
I	Buspirone hydrochloride	Pilocarpine nitrate	Benzbromarone	Dicloxacillin sodium salt		
J	Mesoridazine besylate	Canavanine sulfate monohydrate (L,+)	Trolox	Harmaline hydrochloride dihydrate		
K	Mimosine	Menadione	Clebopride maleate	Dinoprost trometamol		
L	Thiocolchicoside	Diflorasone Diacetate	Clorsulon	Harmane hydrochloride		
M	Ketoconazole	Niridazole	Fusidic acid sodium salt	Ceforanide		
N	Trimetazidine dihydrochloride	Gramine	Parthenolide	Dimethisoquin hydrochloride		
O	Prochlorperazine dimaleate	Methoxy-8-psoralen	Hesperidin	Puromycin dihydrochloride		
P	DO 897/99	Protoveratrine A	Prenylamine lactate	Solanine alpha		

Appendix 2
Prestwick Chemical Compound Plate Layout
(384 well plates)

3

	19	20	21	22	23	24
A	Etanidazole	Sulfanilamide	Butirosin disulfate salt	Hecogenin		
B	Dichlorphenamide	Chrysin	Sulconazole nitrate	Proxyphylline		
C	6-Furfurylaminopurine	Levonordefrin	Avermectin B1	Ebselen		
D	Moxalactam disodium salt	Adrenosterone	Aminophylline	Methylatropine nitrate		
E	Anabasine	Isoetharine mesylate salt	Tetrandrine	Mevalonic-D, L acid lactone		
F	Dimethadione	Nabumetone	Ethaverine hydrochloride	Nisoxetine hydrochloride		
G	Delsoline	Methacholine chloride	Fluorocurarine chloride	Pipenzolate bromide		
H	Ganciclovir	Bacitracin	Ethopropazine hydrochloride	L(-)-vesamicol hydrochloride		
I	Ungerine nitrate	Sulfameter	Napelline	Isopropamide iodide		
J	Rolitetracycline	Ketorolac tromethamine	Equilin	Protriptyline hydrochloride		
K	Hexestrol	Zomepirac sodium salt	Cefmetazole sodium salt	Cinoxacin		
L	Fluvastatin sodium salt	Iodipamide	Methylhydantoin-5-(L)	Esculetin		
M	Trioxsalen	Drofenine hydrochloride	Strophanthidin	Cycloheximide		
N	Phthalylsulfathiazole	(+,-)-Octopamine hydrochloride	Luteolin	(±)-Nipecotic acid		
O	Cephalothin sodium salt	Pridinol methanesulfonate salt	Cefuroxime sodium salt	Amrinone		
P	Capsaicin	Proparacaine hydrochloride	Carbachol	Aminocaproic acid		

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	19	20	21	22	23	24
A	3-Acetamidocoumarin	Oxyphenbutazone	Roxarsone	Quinethazone		
B						
C	Ozagrel hydrochloride	Propoxycaine hydrochloride	Piribedil hydrochloride	Oxaprozin		
D						
E	Asiaticoside	Mesalamine	Betulin	alpha-Santonin		
F						
G	Proguanil hydrochloride	Chlorambucil	Lymecycline	Methiazole		
H						
I	Pivampicillin	(S)-(-)-Cycloserine	Talampicillin hydrochloride	Homosalate		
J						
K	Tribenoside	Nialamide	Rimexolone	Vitamin K2		
L						
M	Risperidone	Rifabutin	Torsemide	Parbendazole		
N						
O	Levocabastine hydrochloride	Clocortolone pivalate	Pyrvinium pamoate	Cyclacillin		
P						

Appendix 3

Plate 1 Data

Rep 1	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
A	40	50	5800	3978	3827.9	3978	3850	3799	3848	4047	4198	3917	4249	4018	4038	4008	4698	4319	4038	4179	4327	4519	3457	30
B	30	160	4048	4317	4489.6	4228	3568	4277	4499	4039	4038	4316	4348	4450	4017	3777	4640	4216	4068	4709	4390	4027	3748	30
C	50	90	3957	3509	3678.6	3358	3969	3647	4249	3388	3769	3888	3917	3987	4229	2049	3800	4027	4698	3697	3997	3589	4467	50
D	50	30	3640	4380	4048	4519	4050	3989	4436	4298	4659	4189	4019	4560	4319	5207	4358	4446	4296	4289	4010	4659	3918	99.9
E	60	80	3718	3749	3879.7	3499	4007	4067	4129	4008	4999	3927	4248	3847	4559	999.3	4599	4597	4388	4208	4467	4369	4667	30
F	79.9	30	4168	3508	3969.7	3769	3988	3989	4100	4647	3897	4498	4448	4048	4708	4779	4589	4170	5588	4219	4417	5106	4658	89.9
G	40	70	4888	3720	3717	4406	3759	4588	4687	3958	3917	4027	4138	4329	4367	4227	4598	3729	4460	4147	4987	4259	5388	70
H	40	110	3797	4157	4037.7	3629	4180	4416	4268	4300	4039	4067	4227	4418	4569	4469	4259	4516	3939	4187	4377	4478	4108	40
I	60	100	4067	4210	3828.9	3899	4168	3669	4107	4378	4496	3690	4446	1839	4559	5237	4348	4569	4398	4298	4457	4306	4208	60
J	40	90	3429	3659	3909.1	4208	4238	3979	3707	4546	3868	4277	5037	1959	3528	4169	3609	4896	4248	4588	4088	4207	3128	50
K	20	160	3580	3968	4128.5	3877	3239	3047	3898	4197	4117	4579	4517	4446	4448	4789	4536	2359	4409	4110	3669	4060	3979	69.9
L	50	200	539.9	3888	3618.7	2169	1959	3687	4020	4188	3990	3799	4028	4337	3410	4687	4079	4280	4378	4500	4088	3628	4037	70
M	30	70	659.7	3548	2939.7	1789	1178	239.9	3629	4218	4117	4638	4157	4139	4397	4288	5069	4158	4260	4228	4258	3849	4307	40
N	40	50	3847	3739	2597.8	3248	3519	4399	4229	4089	4028	4319	3967	3828	3667	4436	4210	4269	3679	4029	4410	3728	3758	60
O	30	70	3879	4287	3659.2	3099	3858	4598	3878	5510	4568	4188	4639	1599	4140	3758	3728	3959	4327	4258	4338	4696	4549	60
P	50	40	4167	4227	3868.7	3529	4279	4099	4437	4378	4588	5117	4256	4100	4910	4889	4189	9188	4479	4669	4567	4409	4986	50

Rep 2	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
A	40	40	7087	3750	13285	4067	4408	4357	4008	4019	4559	4609	3968	4497	4390	4458	4267	4337	4198	3900	4319	4190	3528	20
B	30	110	3698	4189	3967.9	3828	4038	5248	4147	4037	4319	4137	4150	4548	3978	4658	3869	4170	3939	3937	3928	4058	3567	50
C	40	70	3890	3599	3979	4167	3879	3759	4148	4169	4147	4097	3777	4028	4448	2238	3720	4269	3988	3340	4227	3750	3788	80
D	40	60	3230	3769	4017.5	4030	4948	3928	3949	4149	4227	3929	3817	4349	4429	4029	3807	4207	3867	3970	3697	3650	3150	30
E	40	60	3789	3727	3449.2	3520	3929	3979	3910	3937	4307	4078	4168	3878	4199	799.4	4009	4138	4047	4019	5437	3909	4758	40
F	20	100	3617	3969	4100	3928	3570	3958	5337	4277	3568	4250	4150	4499	4446	4568	3778	4600	5937	4500	3959	3818	4847	20
G	50	30	4338	3969	3878.5	3778	4117	4999	4009	4258	4268	4289	4018	3848	4268	4067	4168	3918	3930	3998	4686	3760	4569	20
H	40	70	3510	3748	3589.1	3678	4028	4309	4079	4267	3898	4109	4337	4846	4588	3837	4166	4129	3778	4147	4208	4007	4597	70
I	20	70	3779	3907	4176.9	3680	4519	3957	4338	4480	4517	3970	4380	1599	4498	4287	4278	4307	4028	3799	5985	4148	2829	50
J	40	90	3708	3689	3659.2	4327	3297	3879	4038	4257	4387	4098	4029	1319	3569	4108	3168	4329	3767	4569	4100	4256	4059	60
K	50	50	3748	4238	3779.2	3539	3920	4279	4117	4017	4169	4067	3949	5009	4268	4927	3909	2130	4457	3818	4169	3880	3467	60
L	90	130	190	3630	2168.5	2159	2350	3959	4079	3649	3680	3909	3609	4189	3777	3830	3569	3787	3628	4647	4027	4028	3109	20
M	40	80	509.7	3600	2449.2	1809	1350	489.7	4429	4288	3919	4399	4177	4239	4759	4109	3950	3637	3840	4149	3689	3917	4369	60
N	79.9	120	3750	3419	3249.8	2449	3579	4067	4070	4047	3547	4007	3927	4129	3977	3977	3589	4427	4489	4057	3718	3899	4389	70
O	50	90	3828	3738	3549.1	3547	3830	4500	3730	4997	4509	4058	4129	1079	4428	4348	4450	4629	4576	3949	3948	4387	5230	70
P	0	30	4319	4337	3859.7	4457	4547	4237	4207	4259	4138	4989	4360	4827	3928	4387	4139	9462	4379	4308	4396	4578	4609	30

Appendix 4

Plate 2 Data

Rep 1	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
A	80	70	3619	3468	3627.4	3529	3999	3899	3849	3809	4320	3748	3840	4316	4460	4059	3650	4378	4357	4090	4087	4229	3790	100
B	50	50	3449	4069	3187.6	3547	3748	4150	4017	4137	3689	3998	3858	4050	10053	4127	3489	5127	3909	4098	4077	2289	4299	40
C	30	40	3707	3560	3207.9	3178	4788	3519	3690	3527	3689	3608	4057	3967	4767	4048	3386	4099	4020	3988	3940	3278	3327	79.9
D	90	50	3798	3750	4109.4	3438	3367	4040	3678	7038	4560	4638	4399	3607	4227	4037	5790	3769	4417	3769	4089	3897	4127	70
E	50	40	3548	3868	3238.3	3660	3349	3508	3070	3408	4117	4567	6248	6009	3628	4067	4058	3468	4506	4100	3788	3709	3867	40
F	70	30	3809	3298	3919.1	3747	3517	3579	4007	3838	3817	4589	6684	5808	3959	3458	1299	4207	4007	3820	3428	3779	5106	80
G	60	40	3499	2239	3430	3597	3777	3907	3839	3787	4728	5020	9820	5468	3609	3816	3390	11512	4229	3840	3997	3710	4729	89.9
H	40	120	3639	3219	3367.8	3758	3878	3429	3459	3760	3668	6438	6486	5266	3379	4138	4008	4497	4099	4409	3580	3589	4029	50
I	50	40	3427	2899	3779.7	3909	3670	3688	3557	3529	4048	3627	4289	4498	4237	3449	3979	4058	4300	3767	3940	4177	3028	50
J	40	60	3719	3778	3897.4	3429	3668	3800	2678	2548	3670	4099	3837	4378	3877	3999	3719	4108	3608	4228	3918	3330	2859	80
K	70	40	3938	3518	3627.6	3519	3859	3529	4059	3859	4478	3777	1870	4627	3880	2820	3958	3710	4210	799.6	4076	4169	3649	79.9
L	90	140	4160	3769	3958.9	3990	3948	4068	3469	3870	3938	4409	4137	3717	3868	3708	3747	3768	4198	3997	3777	3969	3288	79.9
M	80	70	3688	3868	3558	2288	3927	3379	3987	3919	7405	4098	3637	4069	4259	3860	539.9	4197	4160	4189	4088	4286	4620	40
N	120	70	4237	3640	3937.7	3857	3588	3390	4259	4366	4060	3767	3657	3657	3788	3440	4097	3598	3859	3760	4368	3939	3808	90
O	60	30	4107	3469	3149.8	3498	3848	4059	3678	4028	4379	4050	3990	2818	3857	4238	3987	3839	3859	4078	4189	4889	4000	60
P	99.9	50	4880	3970	4007.6	4480	9083	4097	4218	4047	1229	4287	4199	4178	4479	4107	3987	4327	3809	4108	4207	4238	3558	60

Rep 2	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
A	50	80	4007	3789	3718.1	3808	3839	4157	3738	3707	3739	4099	4019	4866	4148	4569	4459	4788	4148	3974	4478	5047	4118	80
B	60	190	3979	3468	3548.4	4047	3759	3460	3288	3927	4099	4108	4498	4299	10136	4569	4170	5436	4038	4008	4400	2598	3687	60
C	50	60	3548	3050	3369.2	3578	4488	3587	3539	4359	4019	3937	4207	4577	3960	4359	3298	3437	3908	3537	3730	3718	3567	30
D	40	70	3637	3029	3769.1	3437	3478	3629	3327	8074	4058	4860	5588	4317	4009	4009	3857	4117	3849	3378	3978	3420	3968	60
E	50	60	3467	3788	3289.9	3107	3378	3247	3809	2978	4478	6257	7407	4759	3458	3847	3858	3207	3639	3700	3579	3519	4168	50
F	30	60	3289	3277	3267.9	3128	3609	3647	3658	3918	5166	7069	7198	5258	3478	3219	1079	3918	3819	3769	3489	3757	4238	90
G	60	40	4127	1669	3219	3460	3278	3380	3300	3970	5508	6190	8298	6636	3647	2998	3808	11552	3708	3549	3718	4088	4178	90
H	60	50	3458	3128	3237.7	3498	3499	3109	3329	3388	5170	7806	6476	5669	3819	4179	3477	4318	3407	3557	3969	3817	5209	40
I	20	80	3049	3288	3568.6	3327	3398	3238	3428	3569	4249	4267	3777	4576	3609	3769	4320	4067	3677	3338	4068	3678	3757	80
J	50	130	3239	3508	3599.1	3089	3428	3859	2128	2769	3647	3489	3279	3728	3259	3707	3700	3889	3880	4519	3788	3468	3549	20
K	50	60	3659	3899	3029.3	3949	3650	3888	3137	3100	3667	3830	1979	4337	3777	3397	3419	3609	3510	879.9	3558	3589	3777	40
L	110	180	3338	3228	3368.1	3320	3328	2949	3159	3960	3368	3600	3419	3088	3627	3550	4147	3667	3837	3670	3248	3680	2738	70
M	40	70	3138	3178	3749.9	2419	3450	3888	3689	3608	7318	3767	3368	3819	3968	3339	749.6	3477	4289	3599	3758	3338	4507	60
N	30	90	4048	3089	3447.3	3179	3660	3047	3230	3518	3520	3617	3269	3797	3578	3538	3849	3200	3877	3538	3920	3509	4360	99.9
O	20	9.99	3459	3300	3128.2	3668	3367	3529	3608	3397	3397	3838	3528	3168	3379	3589	3747	3339	3549	3899	3347	4229	4227	50
P	80	70	3790	3670	3569.5	3837	8944	3939	3939	3647	1310	4087	3778	4038	3789	4509	3568	3930	4009	3499	4137	3520	4709	30

Appendix 5

Plate 3 Data

Rep 1	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
A	50	30	3628	3459	3427.5	3910	3948	3589	3739	3847	4230	4686	3998	3508	3957	3408	3957	3618	3498	3590	3630	3570	3298	70
B	20	90	3769	3598	3468.4	3169	3457	3657	3580	3570	3598	3430	3508	1830	10996	3080	4647	3778	3788	3068	3700	3789	3290	40
C	89.9	50	3260	3080	2859.2	2820	3527	3349	3370	3218	3409	2998	3378	2849	3409	3318	2979	4947	3910	3097	3558	2949	2118	40
D	80	80	3068	4057	3887.4	3168	3210	2988	3359	3400	3198	2989	3538	2989	4037	3179	3179	2949	3419	2738	3038	3229	2398	100
E	99.9	60	3137	7439	3307.7	2699	3140	2809	3528	2878	2999	3977	2660	2969	3048	3039	3119	1299	2929	2768	4949	2499	2109	80
F	60	150	3988	5867	3219.6	2999	3059	2808	3310	3118	3098	2779	2838	3418	3299	3140	1240	3707	3460	2679	3227	2988	3380	110
G	30	110	3240	2968	2899.4	1839	3368	3118	3358	3649	3138	3019	2188	3298	2580	3298	2969	11787	5040	3607	3469	3038	4008	70
H	60	100	3288	3568	2828.4	3188	2889	2900	4049	3400	3278	3080	3008	2470	2959	3059	3418	6696	7328	7414	3618	2819	2997	90
I	60	50	3540	3019	3198.6	3029	2898	3400	3639	4157	3130	2609	3457	3389	3210	3307	4127	4099	7146	4078	3719	3378	3079	70
J	20	120	2909	2930	3117.9	3278	3387	3668	2989	3209	3127	389.8	3460	3589	3060	2890	4608	6819	6096	3537	3138	3329	2799	70
K	40	70	3059	3338	2779.7	2660	2940	4407	3247	3659	3608	3159	3148	3068	2998	370	3989	5855	4528	2938	3089	2869	2368	70
L	120	80	3198	3148	3238.2	3548	3718	3178	3570	3257	3459	3430	2909	3170	2730	2978	3529	3137	3399	3029	3418	3078	3718	80
M	60	99.9	3238	3279	3109.2	3508	3669	3408	3129	3197	859.6	3208	3799	3148	2528	3168	699.9	3060	3190	2999	3129	3038	3778	40
N	60	50	3297	2878	3886.8	3568	3507	3429	3457	4639	3907	3089	3655	2958	3098	3020	2878	3239	3059	3158	2977	2810	4057	30
O	40	40	3229	3399	3709.4	3598	3379	3578	3579	5877	3317	3289	3087	3518	3068	3229	3367	3247	3119	3009	3208	3507	3169	90
P	80	50	3649	3747	3958.7	3368	4389	4050	3638	3548	4028	4330	3619	3258	3607	3457	3648	3068	3200	3419	3529	3779	4457	20

Rep 2	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
A	20	30	3548	3520	2969.4	3329	3408	3039	3119	3218	3227	3297	3469	3430	3299	3387	3749	3499	3518	3447	3767	3420	3917	90
B	40	40	3188	2820	3129.9	2898	3230	2888	2528	2848	3290	3117	2919	1510	10144	2628	4157	3169	3108	3247	3647	3588	4356	80
C	90	90	2809	3009	3159.5	2360	2888	2738	2669	2789	2750	2788	2750	2889	2589	3217	2758	2839	3048	2849	3467	2389	4127	50
D	30	60	2830	2990	4137.9	3210	2868	2599	2750	3088	2838	3159	2649	3038	3887	2820	2830	2818	3038	3059	2770	2829	4796	30
E	50	30	5396	6278	3428.1	2619	2568	2418	2480	2650	2678	3230	2629	2579	2748	2639	2569	860	2928	3059	4387	2850	4297	10
F	70	100	4786	3008	2689.4	2248	2328	2819	2699	2559	2718	2520	2608	2949	2639	2518	1189	3659	2718	2589	3129	2999	4789	89.9
G	50	90	2568	2960	2567.9	1679	2390	2319	2748	2899	2539	2899	1950	2809	2708	3018	2698	10100	2698	2639	2729	2849	4880	40
H	50	120	2609	2789	3038.8	2589	2068	2700	3429	2528	2469	2660	2898	2680	2688	2339	3489	5460	5329	3248	2778	2948	4187	60
I	50	50	2909	2408	2727.8	2718	2549	2678	2709	3687	2569	2749	2738	2898	2900	2869	6257	3518	6469	4149	2828	2938	3398	70
J	50	50	2728	2919	2619.4	2639	2699	2569	2570	2620	2588	189.9	2639	3017	2609	2909	4378	5990	5949	3229	3128	2809	3668	70
K	60	70	3079	2938	2359.3	2559	2680	2670	2529	2670	2928	3238	2790	2800	2689	169.9	3028	2859	2838	2789	2829	2999	4239	90
L	50	99.9	2688	2709	2479.4	2400	2880	2469	2959	2709	2758	2620	2579	3070	2699	2730	3058	2729	2819	3238	2989	3129	2799	50
M	150	100	2870	2638	2809.2	2609	2420	3027	2739	3300	949.5	2668	2599	3198	2530	2979	709.6	2938	3018	3147	2909	3218	4539	60
N	99.9	70	2788	2438	3379.1	2500	2618	2710	3030	3879	2678	2868	3450	2639	2910	2589	2859	2499	3059	2848	3358	3028	4588	70
O	50	450	2969	2750	2889.4	2919	2939	2528	2839	3048	3189	2929	2888	3069	2779	2679	2728	3047	3139	2960	3459	3607	4768	50
P	60	70	3570	3380	3337.3	3627	3619	3140	3259	2789	2698	3729	3448	3090	3329	3429	3199	3220	3300	3147	3348	3580	5327	40

Appendix 6

Plate 4 Data

Rep 1	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
A	70	30	3678	3828	3239.8	3359	3217	3399	3548	3468	3409	3859	3349	3247	3559	3538	3620	3238	3518	3299	3569	3739	4968	70
B	70	99.9	3058	3158	3107.7	3419	2948	3258	3077	3177	3118	3140	2949	3049	9909	3299	3318	3408	3258	3447	3299	3370	5197	110
C	40	20	2619	2918	2669.4	2939	2898	2658	2588	2829	2478	2458	3010	4087	2968	2828	3138	3029	2710	3128	2960	3309	4487	40
D	40	60	3018	3179	2857.7	2828	2849	2799	2659	3158	2469	2548	3040	3058	2959	2780	3077	3020	2788	3020	3127	3408	4356	50
E	40	110	2738	2570	2119.7	2739	2528	2278	2459	2888	2720	2399	2770	2559	4457	2658	2600	2570	2678	2619	3217	2889	5878	50
F	50	100	2288	2140	2438.1	2489	2358	2980	2879	2599	2829	2839	2389	2549	2930	2818	2689	2759	2908	2809	2918	3229	5935	50
G	50	40	3278	2949	2348.8	1730	1958	2828	2770	2568	2640	2818	2458	2590	2460	2959	2738	10131	3260	3479	3029	3188	5360	60
H	30	60	2779	2538	2579.8	2678	2990	2879	2720	2868	2609	2708	2848	3109	2679	2758	2748	3028	3028	2799	2768	2958	4857	60
I	50	99.9	2999	2949	2219.5	4369	2919	2699	3029	3118	2608	2679	2950	2940	2938	2460	2979	3337	3037	3658	3068	3918	3898	100
J	70	9.99	2570	2689	3048.5	3128	2358	2559	2509	2988	2638	3009	2869	2579	2868	3238	2668	2710	3119	3418	2938	3018	3859	69.9
K	80	150	2790	2928	2809.8	5905	2520	2829	3859	2808	2648	3149	3008	2730	2659	3299	3129	3400	3990	3009	3038	3520	4268	40
L	80	40	2649	2858	2648.7	2868	2529	2709	2700	2519	2640	2638	2559	2770	2589	2558	2718	2460	3317	4297	3460	3089	4578	20
M	70	50	2739	3138	2669.4	2809	2779	2799	3388	3379	3269	3250	3069	2859	2488	2600	3129	3129	3830	8424	4099	3250	5819	80
N	30	100	2978	2610	2689.4	3059	3098	2808	2648	2778	2479	2739	2459	2968	2750	2869	2909	2738	3319	3467	3440	3149	4988	30
O	40	90	2529	2978	2979.5	2908	3148	3008	3049	3098	3309	3329	3120	3039	3248	3287	3160	2938	3018	3589	2789	3209	5459	60
P	50	80	3797	4037	3457	3708	2939	3578	3409	3368	3350	3529	3577	3477	3388	3509	3059	3439	3809	3730	3768	3750	6398	20

Rep 2	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
A	90	60	3459	3729	3588.9	3648	3609	3648	3718	3499	3770	4157	4018	3597	3510	3720	4097	3420	4410	4368	4189	3707	4806	50
B	280	69.9	3527	3089	3349.7	3367	3788	3219	3359	3640	3159	3628	3489	3799	11098	3558	3499	3728	3887	3639	3578	3708	5185	60
C	50	60	3429	2978	3388.9	3048	2879	3149	2990	3070	3310	3489	3378	4476	3227	3308	3208	3308	3309	3509	3398	3208	4237	140
D	70	60	3108	2699	2887.8	3219	3118	2980	3069	3297	2848	3610	3630	2939	2979	3020	2958	3180	3238	3380	3688	3309	4749	210
E	100	60	2808	2780	2877.8	2748	3119	3179	2539	2848	2779	2749	3120	2979	5157	3416	3498	2898	3108	3199	3478	3479	5619	50
F	40	2048	2729	2858	2987.5	3538	3130	3048	2909	2748	3028	2658	2908	2740	2980	3128	3178	2849	3389	3507	3549	3039	6138	60
G	89.9	89.9	3397	2719	2558.4	1889	2129	2889	3119	3158	2908	2839	3259	3258	3089	3148	3129	7676	3989	3537	3198	3828	4058	50
H	140	110	2638	3219	3078.6	2658	3157	3309	2998	3038	3469	2759	2888	2990	3038	2888	2968	3330	3539	3147	3318	3438	4488	70
I	60	80	3269	3080	2609.8	4968	3247	3189	3068	2907	3129	2848	3289	2868	3319	3268	3088	3217	3427	3277	3707	4188	4017	60
J	70	60	2809	2688	3318.3	2809	2769	3348	2878	2978	3127	2659	2738	3080	3010	3227	3389	3539	3549	3259	3428	3847	4279	50
K	70	260	3198	3289	3189.8	5899	2970	3488	3767	2908	2938	3070	2848	2937	3009	3739	3367	3460	4347	3359	3257	3278	4257	60
L	70	100	2990	3050	2939.2	2979	3030	2940	2889	3209	2769	2969	2789	3180	3268	3028	2869	2838	3148	3588	3769	3458	4599	80
M	20	170	3159	2918	2888.2	3287	3320	3258	3288	3109	3337	3458	3008	3188	2980	3190	3660	3100	3759	8466	4378	3277	3970	70
N	50	150	3059	3019	2858	2978	2920	2858	2588	2999	3367	2959	2868	3249	2770	3008	3038	3339	3129	3488	3219	3360	4629	70
O	30	140	2858	3627	2739	2920	3399	3128	3187	3427	3140	3208	3108	3148	3529	3559	3389	3379	3240	3489	3727	3019	5877	79.9
P	60	70	3570	3380	3337.3	3627	3619	3140	3259	2789	2698	3729	3448	3090	3329	3429	3199	3220	3300	3147	3348	3580	5327	40