NOVEL MONOAMINE OXIDASE B INHIBITOR DOWNREGULATION OF LIPOPOLYSACCHARIDE-INDUCED PRO-INFLAMMATORY CYTOKINES

by

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Abstract

Oral inflammatory diseases (e.g. periodontitis) and gastrointestinal inflammatory bowel diseases (e.g. Crohn’s disease and ulcerative colitis) are characterized by an imbalance in secretion of pro- and anti-inflammatory cytokines leading to inflammation and epithelial and endothelial cell barrier disruption. Monoamine oxidase (MAO) B, a pro-oxidative enzyme, is induced in epithelial cells of a rat periodontal disease model and topical application of a known MAO inhibitor reduced disease (Ekuni et al., 2009). MAO B inhibitors also reduced TNFα secretion in epithelial cells and decreased endothelial cell hyperpermeability associated with inflammation (Tharakan et al., 2010; Whaley et al., 2009). MAO B inhibitors, such as deprenyl, decrease inflammation, however the mechanism by which this occurs is poorly understood. The Putnins laboratory with collaborators have developed reversible and selective MAO B inhibitors derived from deprenyl that do not cross the BBB. These non-BBB permeable novel inhibitors were developed to reduce CNS-based negative side effects, however, their anti-inflammatory effects have yet to be investigated.

Lipopolysaccharide (LPS) is a component of the gram-negative bacterial membrane and is a key mediator of inflammation. The aim of this study is to determine if four novel MAO B inhibitors alter LPS-induced pro-inflammatory cytokine expression in intestinal endothelial and epithelial cells using in vitro cell culture modeling. The two cell lines expressed MAO B but not MAO A protein and the novel MAO B inhibitors did not reduce cell viability nor induce cytotoxicity or apoptosis. However, one compound demonstrated anti-apoptotic effects in intestinal epithelial cells. The novel MAO B inhibitors reduced LPS-induced protein secretion and gene expression of IL-8, IL-6 and TNFα, pro-inflammatory cytokines commonly seen in mucosal inflammatory diseases.

Ultimately, this project aims to provide in vitro evidence for the therapeutic potential of novel MAO B inhibitors for the treatment of LPS-induced pro-inflammatory cytokines. Reduction of pro-inflammatory cytokine secretion and gene expression by novel MAO B inhibitors may pose an effective clinical approach to treat a variety of mucosal inflammatory diseases.
Preface

This dissertation is original, unpublished and independent work by the author M. Tra and represents a project that is part of a collaboration of the Putnins Laboratory and the Centre for Drug Research and Development (CDRD). Thesis preparation was done in its entirety by M.Tra. E. Putnins, as supervisor, and V. Goebeler, as research manager, provided support throughout the project in concept formation, experimental design and thesis edits as required.

All of the work in this thesis was conducted in the Laboratory of Periodontal Biology and CDRD at the University of British Columbia. Research experiments were performed, data were analyzed and figures were composed entirely by M. Tra except for the following items:

Chapter 1. Figures 1 to 8 were used with permission from applicable sources.
Chapter 1. Figure 9. The experiment was performed and data were analyzed by C. Kim from CDRD.
Appendix. The experiment was performed by J. Terc from CDRD and data were analyzed by M. Tra.
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<tr>
<td>AJ</td>
<td>adherens junction</td>
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<tr>
<td>BBB</td>
<td>blood brain barrier</td>
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<tr>
<td>Bcl</td>
<td>B-cell lymphoma</td>
</tr>
<tr>
<td>Caco-2</td>
<td>human colorectal adenocarcinoma cells</td>
</tr>
<tr>
<td>CD</td>
<td>Crohn’s Disease</td>
</tr>
<tr>
<td>CDRD</td>
<td>Centre for Drug Research and Development</td>
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<tr>
<td>Ct</td>
<td>threshold cycle</td>
</tr>
<tr>
<td>CXCR</td>
<td>CXC chemokine receptor</td>
</tr>
<tr>
<td>DSS</td>
<td>dextran sodium sulfate</td>
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<tr>
<td>GI</td>
<td>gastrointestinal</td>
</tr>
<tr>
<td>H$_2$O$_2$</td>
<td>hydrogen peroxide</td>
</tr>
<tr>
<td>IBD</td>
<td>inflammatory bowel disease</td>
</tr>
<tr>
<td>ICAM</td>
<td>intercellular adhesion molecule</td>
</tr>
<tr>
<td>IFN</td>
<td>interferon</td>
</tr>
<tr>
<td>IKK</td>
<td>inhibitory kappa-light-chain-enhancer of activated B cells kinases</td>
</tr>
<tr>
<td>IL</td>
<td>interleukin</td>
</tr>
<tr>
<td>IL-6R</td>
<td>interleukin-6 receptor</td>
</tr>
<tr>
<td>IκB</td>
<td>inhibitory kappa-light-chain-enhancer of activated B cells</td>
</tr>
<tr>
<td>LPS</td>
<td>lipopolysaccharide</td>
</tr>
<tr>
<td>MAO</td>
<td>monoamine oxidase</td>
</tr>
<tr>
<td>MAPK</td>
<td>mitogen-activated protein kinase</td>
</tr>
<tr>
<td>MS</td>
<td>Multiple Sclerosis</td>
</tr>
<tr>
<td>MSD</td>
<td>Meso Scale Delivery</td>
</tr>
<tr>
<td>NF-κB</td>
<td>nuclear factor kappa-light-chain-enhancer of activated B cells</td>
</tr>
<tr>
<td>MPP+</td>
<td>1-methyl-4-phenylpyridinium</td>
</tr>
<tr>
<td>PAMP</td>
<td>pathogen-associated molecular pattern</td>
</tr>
<tr>
<td>PG</td>
<td>prostaglandin</td>
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<tr>
<td>PRR</td>
<td>pathogen recognition receptor</td>
</tr>
<tr>
<td>qRT-PCR</td>
<td>quantitative real-time polymerase chain reaction</td>
</tr>
<tr>
<td>RE</td>
<td>recycling endosomes</td>
</tr>
<tr>
<td>RFU</td>
<td>relative fluorescence unit</td>
</tr>
<tr>
<td>RLU</td>
<td>relative luminescence unit</td>
</tr>
<tr>
<td>ROS</td>
<td>reactive oxygen species</td>
</tr>
<tr>
<td>sIL-6R</td>
<td>soluble interleukin-6 receptor</td>
</tr>
<tr>
<td>SEM</td>
<td>standard error of the mean</td>
</tr>
<tr>
<td>SSAO</td>
<td>Semicarbazide-sensitive amine oxidases</td>
</tr>
<tr>
<td>TACE</td>
<td>TNFα converting enzyme</td>
</tr>
<tr>
<td>TE</td>
<td>trypsin-EDTA</td>
</tr>
<tr>
<td>TJ</td>
<td>tight junctions</td>
</tr>
<tr>
<td>TNFR</td>
<td>TNFα receptor</td>
</tr>
<tr>
<td>TLR</td>
<td>toll-like receptor</td>
</tr>
<tr>
<td>TNFα</td>
<td>tumor necrosis factor alpha</td>
</tr>
<tr>
<td>UC</td>
<td>Ulcerative Colitis</td>
</tr>
<tr>
<td>VCAM</td>
<td>vascular cell adhesive molecule</td>
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This thesis is dedicated to my family.
Chapter 1: Introduction

The human body has an enormous surface area exposed to the exterior environment. The skin is the most visible site exposed to the external environment, however, the interface located at the airways, oral cavity, digestive and genitourinary tract are covered in mucosal tissue, that when combined, is larger than the skin. The gastrointestinal (GI) tract contains the largest mucosal surface and is in continuous contact with various antigens and microorganisms residing in the gut, therefore is critical in innate and adaptive immune regulation (Turner, 2009).

The epithelium is a central mediator between the external environment and the host as it lines the entire mucosa. It is essential for fluid exchange and tissue-specific functions. Beneath the epithelial cells resides the lamina propria, housing blood vessels lined with endothelial cells, controlling the influx of blood and immune cells into tissue sites. Epithelial and endothelial cells of the GI tract are therefore crucial for tissue homeostasis and innate and adaptive immunity of the gut as they are in direct contact with various microorganisms and immune cells, respectively.

1.1 Structure and Function of Mucosal Tissues

The mucosal surface, also termed mucosa, is composed of the epithelium and connective tissue known as the lamina propria (Figure 1). Beginning at the mouth, the epithelium of the GI mucosa starts as non-keratinized, stratified squamous epithelium and transitions to simple columnar epithelium in the stomach and intestinal tract (Peterson and Artis, 2014). Epithelial cells are constantly renewed at a rapid rate of every five to seven days. Between the epithelial cells reside goblet and endocrine cells that secrete mucus, fluid and hormones into the lumen of the digestive tract (Sjolund et al., 1983). Mucin covers the epithelium of the mucosal surface, acting as a lubricant and maintaining the essential “wet” environment of the intestinal interior. The function of the highly hydrated mucus includes the prevention of unwanted large particles such as bacteria from contacting the epithelium, limitation of bulk flow and maintenance of the alkaline mucosal surface thereby controlling the absorptive abilities of the GI tract (Johansson et al., 2008; Linden et al., 2008).
Figure 1. Anatomy and immune cells of the intestinal mucosa
The mucosa is covered by mucus and consists of intestinal epithelial cells and the lamina propria. The muscularis mucosae, submucosa and muscle layer reside below the mucosa. Reprinted by permission from Macmillan Publishers Ltd: Nature Reviews Immunology, Mowat and Agace, copyright 2014.

The lamina propria, located beneath the epithelium, consists of connective tissue housing mucosa-associated lymphatic tissue, blood and lymphatic vessels, which provide access to immune cells, lymphatic drainage and blood and nervous supply for the intestinal tract (Figure 1). The lamina propria is loosely packed, which allows scaffolding of the villi and increases the surface area and absorptive characteristic of the intestinal tract (Mowat and Agace, 2014). Nutrients that are absorbed in the intestinal tract are able to reach other areas of the body through intestinal endothelial cells that line blood vessels of the lamina propria. Endothelial cells also control the entrance of immune cells from the blood stream into intestinal tissue.
The muscularis mucosae is found underneath the mucosa and is a thin layer of smooth muscle that creates the folds of the mucous membrane allowing absorptive cells within the epithelium to be fully exposed to the luminal contents (Figure 1). The submucosa, containing parasympathetic nerves, lined by a thicker muscle layer is situated beneath the muscularis mucosae (Mowat and Agace, 2014).

The intestinal mucosal barrier faces a critical function. It must discriminate between invasive organisms and harmless antigens such as food proteins and commensal bacteria, meanwhile absorbing essential nutrients and digested dietary antigens to maintain tissue homeostasis, or a disease-free state (Dubois et al., 2005; Mowat, 2003). Commensal microbiota exist symbiotically in the GI tract and are essential to human health as they alter luminal pH and may compete against pathogens for nutrients (Artis, 2008; Backhed et al., 2004; Hooper et al., 2001; Lebeer et al., 2010). A constant control of epithelial cell proliferation, apoptosis and low level of inflammation is present in the intestine to limit bacterial penetration and maintain its physiological state (Duerkop et al., 2009; Gersemann et al., 2008; Sansonetti, 2004). In addition, an entire network of cellular components residing in the mucosa orchestrates this fine balance of homeostasis. For example, dendritic cells residing in the lamina propria are able to sample luminal antigens and goblet cells secrete mucus to trap large unwanted particles (Rescigno et al., 2001; Salim and Soderholm, 2011). However, our focus is on the epithelial and endothelial cells of the intestinal mucosa. Since these cell types are critically placed at the forefront of antigen, blood protein and immune cell contact arising from either the lumen or systemic blood stream, they present crucial sites of innate and adaptive immune regulations.

1.1.1 Epithelium

Lining every organ, gland and entire surface of the body, the epithelium is the crucial interface between the moist exterior environment and host. Types of epithelial cells range from simple, stratified, keratinized, squamous, cuboidal and columnar classifications. Specifically in the intestinal tract, epithelial cells, also known as enterocytes, make up eighty percent of the epithelium and form a contiguous lining of uniformly polarized columnar cells that possess a brush border composed of microvilli that line the crypts and villi of the
intestine (Rodriguez-Boulan and Macara, 2014; Turner, 2009). Polarity or asymmetry of the epithelium is fundamental for tissue function and segregates the apical and basolateral membrane domains (Simons and Mlodzik, 2008; St Johnston and Ahringer, 2010). The importance of epithelial cell polarity includes cell shape acquisition, directional transport and spatiotemporal responses from neighbouring cells (St Johnston and Ahringer, 2010). A widely used in vitro cell model that mimics enterocyte-like monolayers is the transformed, heterogeneous, human epithelial colorectal adenocarcinoma cell line (Caco-2) that can form luminal and basolateral compartments without the confounding influence of other cell types of the mucosa (Meunier et al., 1995; Press and Di Grandi, 2008). Other cells within the epithelium include Paneth, goblet and enteroendocrine cells located in the intestinal villi and are involved in innate and bacterial defense and mucin and hormone secretion, respectively. These differentiated specialized cell types are constantly replaced by intestinal pluripotent stem cells residing in proliferative crypts (Bjerknes and Cheng, 2006; Marshman et al., 2002; Rodriguez-Boulan and Macara, 2014).

Under physiological conditions, polarized epithelial cells are linked by junctional complexes and act as a protective barrier that is relatively leaky, enabling paracellular flux of solutes and nutrients. The majority of fluid exchange and size selectivity are controlled by tight junctions (TJ), which are adaptable and regulated by other components of the junctional complex (Fihn et al., 2000). The apical junctional complex consists of the TJ and adherens junction (AJ) supported by actin and myosin filaments. AJs are made of cadherin proteins, and maintain intercellular communication, cellular proximity and are required for TJ assembly (Hermiston and Gordon, 1995). In addition, claudin and zonula occludens proteins are crucial for assembling and maintaining TJs (Fihn et al., 2000; Watson et al., 2005).

The epithelium has an important role in maintaining mucosal and commensal homeostasis as it communicates and modulates underlying immune cells (Cario et al., 2002; Rakoff-Nahoum et al., 2004; Watson et al., 2001). Epithelial cells maintain the mucosal defense system by expression of pathogen recognition receptors (PRRs) and thus, are the backbone of the innate immune system, which will be discussed in detail later (Akira et al., 2006; Medzhitov and Janeway, 2002).
1.1.2 Endothelium

The endothelium lines the entire vascular system and consists of a single layer of endothelial cells, separating blood from underlying tissues. There is phenotypic variation of the endothelium depending on the location in the body but they share similar characteristics and functions. For example, microvascular endothelial cells line smaller vessels such as capillaries in the dermal, cardiovascular and GI regions, while macrovascular endothelial cells, lining arteries and veins, have an additional two layers of tunica surrounding the monolayer of endothelial cells (Sumpio et al., 2002). The endothelium is versatile and multifunctional as it is involved in active signal transduction, thrombosis, thrombolysis, modulating vascular tone and blood flow and regulating immune and inflammatory responses by controlling blood cell interactions with the vessel wall (Bouis et al., 2001; Sumpio et al., 2002; Vita and Keaney, 2002). Similar to epithelial cells, endothelial cells have cell-cell junctions that establish a semi-permeable barrier allowing for active transport of molecules and blood cells (Bouis et al., 2001). However, endothelial cell junctions are less organized than epithelial cell junctions as TJs are intermingled with AJs (Dejana, 2004).

Under physiological conditions, the endothelium maintains an anti-inflammatory and anti-coagulatory state. Vasoactive molecules such as bradykinin and thrombin are released from endothelial cells to alter vessel tone and diameter (Schechter and Gladwin, 2003). Endothelium-derived relaxing factor nitric oxide and prostaglandin (PG) I₂ are key players in keeping the vascular wall in a quiescent state by inhibiting platelet adhesion and aggregation (Forstermann and Munzel, 2006; Furchgott and Zawadzki, 1980; Pober and Sessa, 2007; Sessa, 2004; Stamler et al., 2001). In physiological conditions, endothelial cells do not interact with surrounding leukocytes as they sequester leukocyte interactive proteins and suppress transcription of adhesion molecules such as P- and E-selectin. The endothelium also controls the hyperpolarization and constriction of vascular smooth muscle cells to control vasodilation and modulate blood flow (Busse et al., 2002; Busse and Fleming, 2006; Kinlay et al., 2001; Pober and Sessa, 2007; Saye et al., 1984).

In chronic inflammatory diseases such as inflammatory bowel disease (IBD), patients have reduced barrier integrity allowing increased bacterial penetration of the intestinal epithelium and endothelium (Suenaert et al., 2002). In particular, bacterial virulence factors
like lipopolysaccharide (LPS), a part of the gram-negative bacterial outer membrane, can induce cytokine expression in these cells as they are highly responsive to LPS, further disrupting the epithelial barrier and causing endothelial cell dysfunction (Figure 2) (Cario et al., 2000; Cromer et al., 2011; Dauphinee and Karsan, 2006; Hansson and Libby, 2006).

**Figure 2. Inflammation in intestinal endothelial cells**
Inflammation of the endothelium results in the release of cytokines and chemokines leading to adhesion molecule expression, leukocyte infiltration, increased endothelial permeability and thrombosis. Reprinted by permission from Baishideng Publishing Group Co., Ltd: World J Gastroenterol, Cromer et al., copyright 2011.

### 1.2 Mucosal Response to Lipopolysaccharides

The GI tract is colonized by a vast number of microorganisms, or microbiota, which consists mainly of bacteria though viruses and fungi are also present. Bacteria of the phyla *Actinobacteria, Firmicutes, Bacteroidetes* and *Proteobacteria* dominate human gut-associated microbiota and consist of both Gram-negative and Gram-positive anaerobes (Eckburg et al., 2005). LPS, a well-preserved component of the outer membrane of most Gram-negative bacteria contributing to its structural integrity, is a key mediator in initiating the innate and eventually, adaptive immune responses (Hellstrom et al., 2005; Qin et al.,
$E. \text{ coli, S. typhosa, P.aeuroginosa, K. pneumonia}$ and select pathogens are sources of LPS and are able to synthesize components of the molecule (Raetz, 1990; Rietschel et al., 1994). Elevated plasma levels of LPS are found in patients with diseases associated with mucosal inflammatory barrier disruption such as IBDs, neurodegenerative diseases, inflammatory lung disease and coronary heart failure (Caradonna et al., 2000; Qin et al., 2007; Seehase et al., 2012). LPS is particularly important in IBDs as gram-negative aerobes, mainly $E.\text{coli}$, make up the majority of bacteria, by weight, in the colon (Macfarlane et al., 1997). Since paracellular channels between epithelial cells offer a potential route of LPS penetration and endothelial cells are especially sensitive to the endotoxin, these cell types are important cell types in the innate immune response (Anderson and Van Itallie, 1995).

### 1.2.1 Structure of Lipopolysaccharides

LPS is comprised of a hydrophobic domain called Lipid A or endotoxin and a polysaccharide region (Raetz and Whitfield, 2002). The polysaccharide region contains a core oligosaccharide most proximal to lipid A and a distal O-specific chain (Figure 3a) (Miller et al., 2005). Pathogenic enterobacteria can only survive if this O-specific chain is present as it protects the bacteria against phagocytic uptake. The core oligosaccharide consists of various hexoses that contribute to its structural variability but maintains a beta-deoxy-octulosonic acid residue linking the polysaccharide region to the lipid A domain (Holst et al., 1996; Rietschel et al., 1996). The carbohydrate lipid A moiety is a glucosamine-based phospholipid that anchors the polysaccharide region of LPS to the outer bacterial membrane (Raetz and Whitfield, 2002). Lipid A is a crucial component of Gram-negative bacterium as it makes up 75 percent of its membrane, is strongly agonistic and is the immunostimulatory centre of LPS (Alexander and Rietschel, 2001). Many Gram-negative bacteria, including pathogens, synthesize lipid A species resembling the $E.\text{coli}$-derived moiety (Raetz and Whitfield, 2002). LPS is a pathogen-associated molecular pattern (PAMP) that is specific for toll-like receptors (a class of PRRs) and is a key player in the activation of innate immunity (Medzhitov and Janeway, 2000a, b).
1.2.2 Innate Immune Response

Exogenous (microbial or non-microbial) or endogenous (tissue anomalies) inducers initiate the inflammatory process (Medzhitov, 2008; Medzhitov and Janeway, 1997; Mogensen, 2009). At injured tissue sites, PRRs on epithelial cells and innate immune cells recognize damage-associated molecular pattern molecules (DAMPs) or PAMPs on invading pathogens (Janeway and Medzhitov, 2002; Kumar et al., 2011). PRR-induced signaling works to coordinate the innate immune response (Barton, 2008; Takeuchi and Akira, 2010). Immune cells such as macrophages, dendritic cells, leukocytes and mast cells sense the signals initially detected by PRRs and induce the expression of chemokines, cytokines and vasoactive amines (Gallego et al., 2011). These inflammatory mediators all contribute to the signaling and homing of circulating neutrophils to enter extravascular tissues at the site of injury and clear any dead cells or tissue.
One class of PRRs is the toll like receptors (TLRs), a diverse group of innate immunity receptors, containing an extracellular, transmembrane and cytoplasmic domain (Figure 3b). More than ten TLR protein homologues have been discovered in the human genome (Aderem and Ulevitch, 2000; Medzhitov and Janeway, 2000b; Raetz and Whitfield, 2002). For example, TLR6 or TLR2 in combination with TLR1 recognize lipopeptides, TLR5 detects bacterial flagellin and TLR3, TLR7, TLR8 and TLR9 are classified as anti-viral TLRs (Aderem and Ulevitch, 2000; Alexopoulou et al., 2001; Hajjar et al., 2001; Hemmi et al., 2000). TLR4 is known as the primary LPS receptor and is able to specifically detect Lipid A (Aderem and Ulevitch, 2000; Hajjar et al., 2001; Hayashi et al., 2001; Lien et al., 1999; Medzhitov and Janeway, 2000c; Raetz and Whitfield, 2002; Rock et al., 1998). A variety of cell types including glial cells, macrophages, adipocytes, epithelial cells residing in the lung and GI tract and endothelial cells throughout the body possess TLR4 and respond to LPS (Li et al., 2003; Vereker et al., 2000; Zeuke et al., 2002). Epithelial and endothelial cells are hyper-responsive to commensal PAMPs such as LPS and can detect Lipid A molecules at picomolar levels (Abreu et al., 2003; Aderem and Ulevitch, 2000; Medzhitov and Janeway, 2000c). In fact, it has previously been shown that LPS increases TLR4 cell-surface expression in Caco-2 cells (Abreu et al., 2003).

PAMPs that ligate TLRs result in different effector responses but all lead to nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) activation, thereby triggering the biosynthesis of inflammatory mediators such as pro-inflammatory cytokines (Bes-Houtmann et al., 2007; Raetz and Whitfield, 2002). Cytokine secretion induced by LPS can affect tissue inflammation in a paracrine or autocrine manner.

1.3 Cytokines and the Inflammatory Response

Cytokines are small polypeptides that modulate immune responses, have chemoattractant properties, and interact with other cytokines and chemokines. They possess a short half-life and are secreted and regulated by various cell types (Eckmann et al., 1993b; Scheller et al., 2011; Zhang, 2008). Cytokine families include chemokines, colony-stimulating factors (CSF), interferons (IFN), interleukins (IL), transforming growth factors (TGF) and tumor necrosis factors (TNF).
Cytokines and other mediators play a central role in modulating the intestinal immune system. Bacterial stimulation induces cytokine expression from immune, epithelial and endothelial cells residing in the gut and are released during acute and chronic inflammation to signal neighbouring immune and inflammatory cells (Rogler and Andus, 1998). In fact, intestinal epithelial and endothelial cells in response to LPS express a myriad of cytokines (Ogawa et al., 2003; Savidge et al., 2006). In addition, along with increased mucosal permeability, a characteristic of patients suffering from IBD is the penetration of bacterial components like LPS to induce an imbalance of pro- and anti-inflammatory cytokines, where expression of certain cytokines can distinguish Crohn’s disease (CD), Ulcerative Colitis (UC) and diverticulitis (Medzhitov, 2008; Rogler and Andus, 1998). Cytokines can further potentiate the disruption of the intestinal epithelial barrier leading to chronic mucosal inflammation (Ivanov et al., 2010). Cytokine expression can be controlled through gene transcription or storage release at the cell surface.

1.3.1 Pro-Inflammatory Cytokine Gene Expression and Secretion during Mucosal Inflammation

The pathological response of IBD is cytokine-mediated inflammation induced by penetration of bacterial components, leading to epithelial apoptosis and disruption of the intestinal barrier (Danese, 2012). Inflammatory progression involving the innate and adaptive immune systems leads to expansion of the intestinal microvasculature and increased IL-8, IL-6, IL-23, IL-12, TNFα plasma levels (Cromer et al., 2011). In fact, IL-6, IL-1 and TNFα are key inflammatory mediators and are elevated in most inflammatory states (Scheller et al., 2011; Zhang, 2008). Expectedly, current therapies target the excessive activity of the immune system induced by pro-inflammatory cytokine secretion. For instance, therapies targeting IL-6 in early onset of CD decreases disease severity and potential development of colon cancer (Foran et al., 2010; Li et al., 2010; Matsumoto et al., 2010; Mudter and Neurath, 2007). Reducing pathogenic T-cell activation and its effects mediated by pro-inflammatory cytokines is crucial at preventing endothelial disruption and hyperpermeability (Danese, 2012). However, targeting one inflammatory mediator may result in decreased effectiveness, eventual loss of response in patients and possibly pro-inflammatory cytokine activation.
We will be focusing on four key cytokines, IL-6, IL-8, TNFα and IL-1β. These cytokines have an important implication in inflammatory diseases including IBD, are induced by LPS in intestinal epithelial and endothelial cells and are regulated partly by NF-kB activation.

1.3.1.1 IL-8 Role in Inflammation

IL-8 is a prototypical chemokine that attracts neutrophils and T-lymphocytes, contributing to the first line of defense in inflammation (Baggiolini et al., 1997). It activates polymorphonuclear leukocytes, which in turn regulate local intraepithelial cells, lymphocytes, neutrophils and macrophages that reside in the lamina propria. IL-8 is also involved in the chemotaxis of basophils, angiogenesis and initiation of the acute inflammatory response (Eckmann et al., 1993b).

IL-8 signaling is initiated by the activation of two G-protein-coupled receptors, CXCR (CXC chemokine receptor)-1 and -2, expressed on the surface of neutrophils (Zeilhofer and Schorr, 2000). After binding to IL-8, CXCR-1 and -2 are internalized and recycled on the cell membrane, thereby acting as its own agonist. Receptor internalization is a fundamental step of chemotaxis as it continuously activates immune cell influx. Since CXCR2 is more rapidly desensitized and internalized, CXCR1 seems to be the more important receptor of IL-8 during the inflammatory process.

Intestinal epithelial cells secrete IL-8 and elevated levels are found in colonic epithelial cell lines treated with LPS as well as serum of UC and CD patients (Daig et al., 1996; Nielsen et al., 1996; Schuerer-Maly et al., 1994). Other cell types such as keratinocytes, bronchiolar epithelial cells, gastric carcinoma cells, macrophages and fibroblasts release IL-8 as well (Schuerer-Maly et al., 1994). Caco-2 cells, a cell-line used extensively over the past twenty years as an in vitro model of the intestinal epithelium, express a substantial amount of IL-8, which is one of the only detectable chemokines in this cell line (Eckmann et al., 1993a; Huang et al., 2003; Schuerer-Maly et al., 1994). Transcriptional and translational mechanisms are involved in the regulation of the production and secretion of IL-8 and vary between epithelial cells.
1.3.1.2 IL-6 Role in Inflammation

IL-6 is a cytokine induced during the acute phase response and is involved in the transition from innate to acquired immunity (Rogler and Andus, 1998; Scheller et al., 2011). The cytokine promotes monocyte differentiation to macrophages and synergizes its effects with IL-23 to skew T-lymphocyte differentiation into Th1 or Th17 T-cells (Danese, 2012; Marx, 2007; Weaver et al., 2007). IL-6 enhances T-cell survival and the resistance to apoptosis at inflamed sites by inducing the overexpression of anti-apoptotic factors such as B-cell lymphoma (Bcl)-2 and Bcl-extra-large on cell membranes (Neurath et al., 2001). Vascular permeability is also induced by IL-6 leading to the release of additional IL-6, IL-1β and TNFα, promoting neutrophil attraction (Gornikiewicz et al., 2000).

IL-6 signaling is initiated once it binds to the gp130-IL-6 receptor (IL-6R) complex in classical signaling or soluble IL-6 receptor (sIL-6R) and subsequently gp130 in trans-signaling (Figure 4). In trans-signaling, although sIL-6R is only found on a few cells, gp130 is detected on the surface of all body cells and can be activated by the IL-6-sIL-6R complex. Therefore, a broad range of IL-6 signals mediated by this complex can activate gp130-positive cells (Jones et al., 2005b; Mudter and Neurath, 2007). Activation of gp130 leads to homodimerization and signal initiation of trans-signaling that promotes T-cell proliferation (Chen et al., 2006). This process is likely mediated at the transcriptional level by activation of the preformed transcription factor and cofactor (Gornikiewicz et al., 2000).

Figure 4. IL-6 classical and trans-signaling
Classic signaling in cells that express gp130 and IL-6R are responsive to IL-6 (left). Cells that do not express IL-6R but gp130 are responsive to IL-6-sIL-6R through trans-signaling (right). Reprinted from Biochimica et Biophysica Acta (BBA) – Molecular Cell Research, Vol. 1813, Authors: Jürgen Scheller, Athena Chalaris, Dirk Schmidt-Arras, Stefan Rose-John, Title: The pro- and anti-inflammatory properties of the cytokine interleukin-6, Page No. 878-888, Copyright 2011, with permission from Elsevier.
Major sources of IL-6 are macrophages but keratinocytes, bronchiole epithelial cells, gastric carcinoma cells and endothelial cells express IL-6 as well (Rogler and Andus, 1998; Schuerer-Maly et al., 1994). IL-6 and sIL-6R are elevated in serum and mucosal biopsies of IBD and septic patients (Gornikiewicz et al., 2000; Rogler and Andus, 1998). Out of all pro-inflammatory cytokines, it has the highest predictive value of CD diagnosis since CD is dominated by the Th1 cell phenotype (Atreya and Neurath, 2005). Not only is the protein increased, elevated IL-6 mRNA levels are found in dextran sodium sulfate (DSS)-induced mouse colitis models and in colonic mucosal biopsy specimens from CD and acute inflammation (Naito 2004). Expectedly, therapeutic blockade of IL-6 as a treatment of chronic inflammatory diseases is currently utilized.

1.3.1.3 TNFα Role in Inflammation

One of the most influential pro-inflammatory cytokines is TNFα. It is known as a key regulator of the inflammatory response as it induces the expression of an entire spectrum of adhesion molecules and cytokines in vascular endothelial cells such as IL-8, intercellular adhesion molecule (ICAM-1), vascular cell adhesion molecule (VCAM-1), monocyte chemoattractant protein-1 (MCP-1), E-selectin and PGI2 (Zhang, 2008). This leads to leukocyte adhesion, transendothelial migration, vascular permeability, vasodilation and thrombosis (Bradley and Pober, 1996; Bradley et al., 2008; Cromer et al., 2011; Mark et al., 2001; Petzelbauer et al., 1994; Rollins et al., 1990). The release of TNFα is a normal response to infection and was originally identified as the LPS-induced humoral mediator, however inappropriate and excessive production of the cytokine leads to a harmful inflammatory response (Bradley et al., 2008; Cerami et al., 1985). TNFα has a role in parasitic and viral infections such as malaria and contributes to carcinogenesis by promoting proliferation, invasion and metastasis of tumour cells (Moore et al., 1999; Rudin et al., 1997).

Pro-TNFα, the precursor form of TNFα, is a 26-kDa membrane-bound protein that is processed by TNFα converting enzyme (TACE) and is secreted as the soluble, mature, 17-kDa form (Bradley et al., 2008; Moss et al., 1997). Both forms are involved in immune response but there is increasing evidence that pro-TNFα may be superior as local concentrations may be high enough to play an important physiological role even if
production of the soluble protein is low (Imaizumi et al., 2000). Soluble TNFα interacts with two receptors, TNFα receptor (TNFR) 1 and 2 (Wallach et al., 1999). Pro-inflammatory and apoptotic processes that lead to tissue injury are usually mediated by TNFR1 and mice that are deficient of the receptor are resistant to lethal doses of LPS (Alexander et al., 2008). TNFR2 can activate endothelial and epithelial tyrosine kinases that mediate cell adhesion, migration, proliferation and survival. These receptors are processed by TACE, which demonstrate the importance of the enzyme in inflammation (Wang et al., 2003).

Cellular sources of TNFα include basophils, B-lymphocytes, astrocytes and keratinocytes but the primary sources of the pro-TNF protein are macrophages and T-lymphocytes (Bradley, 2008; Imaizumi et al., 2000; Kock et al., 1990). They are also found in natural killer cells, mast cells, neutrophils, smooth and cardiac muscle cells, fibroblasts, epithelial and endothelial cells (Bradley, 2008). TNFα and TACE mRNA are found in unstimulated human umbilical vein endothelial cells (HUVEC) and are found to have TACE activity (Imaizumi et al., 2000). Serum and tissue levels of TNFα are elevated in patients with inflammatory and infectious conditions and an increase in TNFR1 and 2 are found in patients suffering from psoriasis (Ettehadi et al., 1994; Nurnberger et al., 1995; Robak et al., 1998).

Currently, TNFα blocking agents are used to treat rheumatoid arthritis, ankylosing spondylitis, CD and other inflammatory diseases (Bradley, 2008). These drugs have different binding and pharmacokinetic profiles and have been shown to be effective at inducing remission, promoting healing and reducing the need for colectomies in CD, at least in short term (Lawson et al., 2006). However, adverse events have already been associated with approved anti-TNFα agents such as development of bacterial infections, tuberculosis, opportunistic infections, demyelinating syndromes, systemic lupus and lymphomas (Sandborn et al., 2004).

1.3.1.4 IL1β Role in Inflammation

IL-1β is a pro-inflammatory cytokine involved in pain, acute and chronic inflammation and autoimmune disorders. Endothelial cells, macrophages, neutrophils, intestinal epithelial cells and natural killer cells secrete IL-1β and increased levels are found
in inflammatory lesions of patients with IBD (Rogler and Andus, 1998). Broadly, its functions include induction of IL-2 release for thymocyte proliferation, B-lymphocyte maturation and proliferation, activation of inflammatory cells and production of prostaglandins, a mediator of fever stimulation, similar to other pyrogenic cytokines such as IFNγ, IL-6 and TNFα (Ren and Torres, 2009). IL-1β has an important homeostatic function but its over-expression leads to pathophysiological changes in disease states like IBD, rheumatoid arthritis, vascular disease, multiple sclerosis (MS) and Alzheimer’s disease (Braddock and Quinn, 2004; Dinarello, 2004). Pro-nociceptive mediator upregulation in chronic pain states is also associated with an increase in IL-1β (Ren and Torres, 2009).

The inflammasome, an intracellular multi-protein complex, is an important regulator of inflammation and is involved in IL-1β activation (Martinon and Tschopp, 2007). It contains nucleotide-binding oligomerization domain (NOD)-like receptor proteins and acts as an activating scaffold for pro-inflammatory caspases that activate pro-IL-1β to IL-1β (Mariathasan and Monack, 2007; Tschopp et al., 2003). For example, activated caspase 1 and matrix metalloproteinase proteins (MMPs) within the scaffold process pro-IL-1β to the bioactive IL-1β form (Kawasaki et al., 2008; Ren and Torres, 2009). IL-1β is recognized by two receptors, IL-1 receptor type 1 (IL-1RI) and type 2. Upon binding to IL-1RI, the IL-1 receptor accessory protein (IL-1RAcP) is recruited to the cell membrane to form the binding receptor complex, initiating intracellular signaling (Rogler and Andus, 1998).

### 1.3.2 Regulation of Pro-Inflammatory Cytokines by Lipopolysaccharide

A defective barrier in IBD allows the paracellular flux of LPS and other luminal antigens to increase intestinal TJ permeability and propagate inflammation (Andreasen 2008, Marshall 2002, Lambert 2009). Although plasma levels of LPS fall within the range of 2-10 ng/mL, the concentration of LPS is greatest in the gut lumen where levels as high as 1.8 µg/mL can be found (Andreasen et al., 2008; Ge et al., 2000; Sharma et al., 2007; Yagi et al., 2002; Yu et al., 2005).

LPS increases TLR4 mRNA and Caco-2 TJ permeability (Guo et al., 2013). TLR4 along with CD14 and MD2, form a receptor complex having an important role in recognizing LPS/LPS-binding protein (LBP) since CD14 transfers LPS to TLR4/MD2 (Figure 3B)
Abreu, 2010; Brito et al., 2004; Triantafilou and Triantafilou, 2005). TLR4 is actually a regulator of CD14 expression in enterocytes (Lizundia et al., 2008). LPS is known to activate CD14 negative cells such as vasculatory endothelial and epithelial cells, however most are known to already express TLR4 (Alexander and Rietschel, 2001; Vita et al., 1997; Zhang et al., 1999).

A key pro-inflammatory effect of LPS is the induction of mediators such as TNFα, IL-1, IL-6, IL-8 and IL-12 from cells involved in the inflammatory process (Alexander and Rietschel, 2001). LPS increases constitutively expressed adherence molecules, shedding of IL-6R, production of sIL-6R and IL-1β in inflamed tissue and thereby thermal hyperalgesia (Atreya and Neurath, 2005; Chessell et al., 2005; Menetski et al., 2007; Sumpio et al., 2002). The E.coli-derived endotoxin also had lethal effects in mice through an increase in TNFα levels, whereas anti-serum to TNFα protected mice from lethality (Bradley, 2008).

Pro-inflammatory activation of cell types such as human endothelial cells induced by LPS result in the release of IL-8 thereby promoting neutrophil adhesion and extravasation (Heidemann et al., 2006; Medzhitov and Janeway, 2000b). Specifically, intestinal microvascular endothelial cells exhibit a strong immune response to LPS and release IL-6, IL-8, VCAM-1 and ICAM-1 (Ogawa et al., 2003). It has also been found that LPS induces an increase of TNFα mRNA in human umbilical vein endothelial cells (Imaizumi et al., 2000). The inflammatory immune response is also triggered in intestinal epithelial cells by the endotoxin (Savidge et al., 2006). Enteric epithelial cells upregulate steady-state IL-8 mRNA and secrete IL-8 in response to LPS stimulation and in some cases, epithelial tumors may proliferate due to the generation of IL-8 (Eckmann et al., 1993a; Schuerer-Maly et al., 1994).

With the requirement of the coordinated interaction of LPS-binding protein, CD14, TLR4 and MD2, LPS stimulation of both epithelial and endothelial cells leads to an increase in pro-inflammatory cytokine production. It is well known that this process happens through activation of NF-κB transcriptional activity and subsequently secretion of the pro-inflammatory signaling molecules.
1.3.3 Regulation of Pro-Inflammatory Cytokine Gene Expression and Secretion

1.3.3.1 Regulation of Pro-Inflammatory Cytokine Gene Expression

The production of pro-inflammatory cytokines is initiated by a well-known transcription factor NF-κB and therefore activation of NF-κB plays a role in controlling inflammatory diseases. NF-κB is ubiquitously expressed and regulates cytokine and immunoglobin gene expression involved in the acute inflammatory response (Blaecke et al., 2002; Liboni et al., 2004). Members of the mammalian NF-κB family include two types: Class I p50, p52 and p65 or Class II RelB and cRel. They all share Rel-homology, essential for DNA binding and dimerization and positively regulating gene expression and transcription of chemokines and adhesion molecules (Huang et al., 2003; Jones et al., 2005a; Morgan and Liu, 2011). They are kept as inactive p50/p65 heterodimers in the cytoplasm by endogenous inhibitor molecules of κB (IκB) that mask the DNA-binding domains of NF-κB (Blaecke et al., 2002; Jobin and Sartor, 2000).

NF-κB is activated by extracellular stimuli such as LPS, reactive oxygen species (ROS), hydrogen peroxide (H₂O₂) or cytokines themselves (Chariot, 2006; Prince et al., 2006; Viatour et al., 2005). Such stimuli lead to activation of IκB kinases (IKKs), which regulate IκBs through phosphorylation (Haddad and Land, 2002). Phosphorylation of IκB leads to IκB ubiquitination and degradation, unmasking the DNA binding activity of the NF-κB heterodimer (Bonizzi and Karin, 2004; Hayden and Ghosh, 2008; Huang et al., 2003; Vallabhapurapu and Karin, 2009). Once free, NF-κB is able to translocate into the nucleus to bind to DNA sequences with NF-κB binding sites and induce the transcription of gene products involved in the inflammatory response such as TNFα, IL-1, IL-8, IL-6, ICAM-1, VCAM-1 and E-selectin (Chariot, 2006; Schmidt et al., 2006; Viatour et al., 2005). Specifically, an example of NF-κB activation induced by mediators that bind the TNFR1 receptor is shown in Figure 5. The TNF receptor type 1-associated death domain protein (TRADD) is stimulated to recruit TNF receptor-associated factor 2 (TRAF2) and protein kinase RIP. TRAF2 in turn recruits IKK and RIP activates the IκB kinase, thus phosphorylating IκB and eventually activating NF-κB as described above.
NF-κB-activated transcription is maintained by continuous degradation of IκB (Haddad and Land, 2002). Mediators like LPS induce expression of IL-8, IL-6 and early response cytokines such as TNFα and IL-1β which can also activate NF-κB (Romier et al., 2008; Salmi and Jalkanen, 2001). It is found that LPS-induced IL-6 transcription is primarily mediated by NF-κB activation (Gornikiewicz et al., 2000). However, IL-8 and TNFα transcription seems to be controlled not just through NF-κB but through possible cross-talk among several pathways such as mitogen-activated protein kinase (MAPK) which affects the rate of cytokine mRNA decay (Haddad and Land, 2002; Huang et al., 2003; Jijon et al., 2002). There is evidence that NF-κB may play a role in LPS modulation of Caco-2 permeability through the increase of TLR4 expression in certain cells and unsurprisingly, increased NF-κB activity is found to be associated with IBDs.

1.3.3.2 Regulation of Pro-Inflammatory Cytokine Secretion

Regulated and constitutive exocytosis are two mechanisms of pro-inflammatory cytokine secretion (Figure 6) (Stanley and Lacy, 2010). In regulated exocytosis, cytokines are packaged in vesicles in the golgi and secreted during receptor-mediated release (Jolly and Sattentau, 2007; Moqbel and Coughlin, 2006; Stinchcombe and Griffiths, 2007).
In contrast, constitutive exocytosis releases cytokines rapidly through tubulovesicular structures called recycling endosomes (RE) and secreted vesicles. This process may be initiated by either receptor stimulation or nuclear DNA transcription and RNA translation (Stow et al., 2009; Stow et al., 2006). Most epithelial cells contain REs and utilize constitutive exocytosis, more specifically, polarized protein trafficking where cargo is transported to the plasma membrane for release within minutes or hours of protein synthesis. This process is best defined in macrophages that package and secrete TNFα and IL-6 (Manderson et al., 2007; Murray et al., 2005). Polarized protein trafficking is dependent on the cytoskeleton since it has the ability to crosslink receptors and contributes to cell-cell communication. The release of cytokines in a polarized manner occurs toward invaginations known as phagocytic cups and filipodia (Kay et al., 2006; Manderson et al., 2007; Murray et al., 2005; Stow et al., 2006). TACE is concentrated in phagocytic cups, allowing for the fusion of REs and catalysis of soluble TNFα formation from its precursor protein (Figure 5). IL-6 and IL-8 are processed in this way as well, but the difference between TNFα and IL-6 or IL-8 is the separation of the cytokines in sub compartments of REs at the golgi since TNFα has a membrane-bound precursor and IL-6 and IL-8 are generated as a soluble cytokine.
(Manderson et al., 2007). Secretion of IL-1β is characterized as non-classical as it is generated as biologically inactive pro-IL1β and synthesized directly into the cytoplasm where it is cleaved by caspase-1 at intracellular sites.

Polarized protein trafficking inducing TACE activity is initiated by mediators such as ROS, LPS and exogenous H₂O₂. These mediators upregulate TACE activity within 30 minutes without changing the cell surface expression of the enzyme and activate intracellular signaling pathways downstream. ROS directly acts on the cysteine switch of the TACE pro-domain which nullifies its inhibitory effect on TACE (Zhang et al., 2001). TACE activity may cross talk with other pathways such as MAPK as selective p38-MAPK inhibitors are able to attenuate LPS-induced TACE activity (Scott et al., 2011).

Currently, anti-TNF therapies tend to bind and neutralize soluble TNFα. It is effective against symptoms of rheumatoid arthritis but not as successful for CD patients (Bradley, 2008; Feldmann and Maini, 2001; Horiuchi et al., 2010). Low molecular mass, long lasting, orally bioavailable inhibitors of TACE activity are presently desirable in the treatment of chronic diseases (Muruńkar et al., 2010). However, each cytokine must be individually evaluated as novel targets for treatment of inflammatory diseases since they each have a distinct trafficking pathway and differ drastically between cell types.

1.4 MAO General Introduction

Monoamine oxidase (MAO) B, a pro-oxidative enzyme, was increased in a LPS-induced rat periodontal disease model (Ekuni et al., 2009). Topical application of an MAO inhibitor reduced disease characteristics such as TNFα and ROS serum levels, however, the mechanism is currently not understood. To date, no systematic review of MAO inhibition affecting a broader scope of cytokine expression exists. MAO enzymes are involved in the following reaction:

\[
\text{MAO} \quad \text{RCH}_2\text{NH}_2 + \text{O}_2 + \text{H}_2\text{O} \rightarrow \text{RCHO} + \text{NH}_3 + \text{H}_2\text{O}_2
\]

Specifically, MAOs catalyze the oxidative deamination of a variety of biogenic amines including neurotransmitters such as serotonin, histamine, and catecholamines like dopamine, noradrenaline and adrenaline (Tipton et al., 2004). Other products of the reaction...
include the oxidative stress molecule $\text{H}_2\text{O}_2$, an aldehyde, either ammonia or a substituted amine (depending on the type of amine that is oxidized) and ROS. Two isoenzymes of MAO exist and are designated as MAO A and MAO B both of which are present in most mammalian tissues (Tipton et al., 2004). The two isotypes are distinguished by their specificity of substrate binding (dependent on substrate concentration and turnover rate) and sensitivity to MAO inhibitors (Tipton et al., 1987). MAO A has higher affinity to biogenic amines such as serotonin, norepinephrine and dopamine and is inhibited by clorgyline while MAO B has a higher affinity to phenylethylamines and benzylamines and is inhibited by selegiline or (-)-deprenyl (Youdim et al., 2006).

MAO A and B have 70% identical protein amino acid sequence and identical intron-exon organization but are encoded on adjacent genes of the X chromosome and have different core promoter regions (Wong et al., 2002). There are species to species variation of the metabolic processes that MAOs are involved in but it is known that the enzymes are highly conserved (Fornai et al., 1999). For instance, the amino acid sequence in rats and humans, have over an 87% identity for MAO A and 88% for MAO B, which demonstrates the evolutionary pressures to maintain specific physiological function of each protein in various species (Bach et al., 1988).

Both isotypes are associated with the mitochondrial outer membrane and microsomal fraction and are found in specific regions of the brain and in certain peripheral tissues (Youdim et al., 2006). Catecholaminergic neurons in regions of the hypothalamus and substantia nigra contain MAO A, serotoninergic neurons and astrocytes contain MAO B while both are located in the endoneurial vessel endothelium, Schwann cells and neuronal axons (Matsubayashi et al., 1986; Westlund et al., 1985; Youdim et al., 2006). Although it is well established that MAOs reside in the brain, we are focused on MAO in peripheral sites. MAO A and B are found in areas such as the intestine, liver, lungs and placenta and function to protect the body by oxidizing biogenic amines from the blood or to prevent their entry into circulation (Youdim et al., 2006). Specifically, both isotypes are found in fibroblasts, capillary and lymphatic endothelial cells, the muscle layers of arteries and veins, pneumocytes, epithelial and smooth muscles cells of the lung and the duodenal mucosa (villi and crypts) and muscularis externa (Edelstein and Breakefield, 1986; Rodriguez et al., 2001;
MAO A is found in more abundance in cardiomyocytes and reproductive tissue while MAO B is abundant in the human liver, platelets, lymphocytes, renal tubules and hepatocytes (Coccini et al., 2009; Raddatz et al., 1995; Sivasubramaniam et al., 2003). In addition, MAO B is not only found in the mitochondria but also in exosomes and is more abundant in cells within the mucosa and submucosa of the duodenum (Prunotto et al., 2013).

We are focused on the effect of specifically MAO B as it has been implicated in many diseases and physiological processes like aging, stress and in neurodegenerative diseases such as Parkinson’s disease, Alzheimer’s disease and amyotrophic lateral sclerosis (Doyle et al., 1996; Hotamisligil et al., 1994; Kennedy et al., 2003; Mallajosyula et al., 2008; Nicotra et al., 2004; Riederer et al., 1983; Sano et al., 1997). It has been speculated that a variety of mechanisms may increase MAO B and induce disease. For instance, elevated MAO B converts 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) to its toxic form 1-methyl-4-phenylpyridinium (MPP+) in glial cells inhibiting mitochondrial electron transport and overall upregulation of oxidative and inflammatory stress through oxygen radical formation (Dauer and Przedborski, 2003; Gerlach et al., 1996; Hauptmann et al., 1996; Mattson and Liu, 2003; Riederer et al., 1983). MAO B inhibitors have been widely shown to have neuroprotective effects that will be discussed further.

However, not only is MAO B upregulated in diseases related to brain tissue, MAO B was found to be one of the top ten genes that were upregulated in a rat periodontitis model induced by LPS and increased 6-fold in disease tissues compared to healthy tissue (Ekuni et al., 2009). MAO B is also upregulated in intestinal tissue in a DSS-induced colitis mouse model (data not shown). Increasing interest has been directed toward determining the effect of MAO B inhibitors to treat non-neural diseases.

1.4.1 MAO Inhibitors

The first MAO inhibitors that were developed, iproniazid and phenelzine, were used as antidepressants as they had psychoenergizing effects by increasing levels of dopamine, noradrenaline and serotonin in the brain (Dostert et al., 1989; Pletscher, 1991). However, non-selective MAO inhibitors exhibited numerous adverse side effects such as liver toxicity,
hypertensive crisis, hemorrhage and sometimes death. Hypertensive crisis, also known as the cheese reaction, has been extensively studied and is a main reason why many non-selective MAO inhibitors have been withdrawn from use. MAO A in the intestinal tract metabolizes tyramine from foods such as wine and cheese. When MAO A is inhibited, tyramine displaces various monoamines such as noradrenaline from neuronal storage vesicles, triggering vasoconstriction and causing the phenomenon of the cheese reaction (Figure 7) (Youdim et al., 2006). Since intestinal MAO A mostly metabolizes tyramine and MAO B does not, the use of selective MAO B inhibitors such as deprenyl has been exploited as treatment for neurodegenerative diseases, as it does not promote the harmful cheese effect (Hasan et al., 1988; Youdim and Weinstock, 2004).

**Figure 7. The cheese reaction**

Inhibition of MAO A allows tyramine from wine and cheese to displace noradrenaline from neuronal storage vesicles, triggering vasoconstriction and causing the Cheese Reaction. Reprinted by permission from Macmillan Publishers Ltd: Nature Reviews Neuroscience, Youdim et al., copyright 2006.
1.4.1.1 MAO-B Inhibitor (-)-Deprenyl

Deprenyl (also known as selegiline) is a selective, irreversible MAO B inhibitor with 500-fold selectivity over MAO A (Magyar, 2011). Similar to many MAO inhibitors, it blocks the metabolism of biogenic amines, possesses antioxidant effects, lowers H$_2$O$_2$ and ROS levels and increases the concentration of dopamine (Carrillo et al., 1991; Hornykiewicz, 2001, 2002). Deprenyl has been shown to hold a large spectrum of various pharmacological activities such as anti-apoptotic and anti-tumour properties (ThyagaRajan and Felten, 2002).

Well-known for its effectiveness on neurodegenerative diseases, deprenyl has been used for the treatment of Parkinson’s disease although the underlying therapeutic effects are not fully understood (Parkinson Study, 2005). Disease progression is slowed by increased dopamine levels, decreased dopaminergic neurodegeneration and age-related MAO B (Green et al., 1977; Youdim et al., 2006). In addition, deprenyl exhibits neuroprotective effects by reducing the toxicity of MPP+, a product of MAO B activity (Sharma et al., 2003).

Deprenyl improves disease characteristics possibly through its anti-apoptotic properties. For example, deprenyl is able to decrease peripheral tissue damage from cardiac failure possibly by decreasing H$_2$O$_2$, increasing Bcl-2 and Bax levels and activating anti-apoptotic protein kinases (Qin et al., 2003; Weinreb et al., 2004). Deprenyl’s anti-apoptotic properties have also been demonstrated in a dose-dependent manner in rat kidney after ischemia reperfusion (Kiray et al., 2008; Tatton et al., 2002; Toronyi et al., 2002). However, there have been reports that at higher concentrations, the protective effects of deprenyl are lost and the drug becomes pro-apoptotic, shown by the increase of caspase 3 with treatment of a higher drug dose (Magyar and Szende, 2004; Seymour et al., 2003; Szende et al., 2000).

Interesting novel effects of deprenyl treatment are the reduction of hyperpermeability of endothelial cells and increase in cell-to-cell adhesion (Jenei et al., 2005). Treatment with the MAO B inhibitor has shown to attenuate vascular endothelial cell permeability induced by serum from burn patients, decrease mitochondrial ROS levels and decrease mitochondrial membrane potential, suggesting anti-apoptotic properties of the drug (Whaley et al., 2009). In lung microvascular endothelial cell cultures and blood vessels of rats, deprenyl also inhibited hemorrhagic shock-induced permeability, cytochrome C release and caspase-3 activation (Tharakan et al., 2010).
1.4.1.2 MAO Inhibitor Transcriptional Effects

In addition to its effects on MAO activity, MAO inhibitors also affect transcriptional processes. PF9601N, a MAO B inhibitor, reduces MPP+ induced cell death and decreases caspase-3 activation by decreasing nuclear translocation of p53 into the nucleus, a key step in apoptosis initiation (Sanz et al., 2008). Phenelzine has no effect on cell viability and NF-κB translocation contrary to tricyclic antidepressants and selective serotonin reuptake inhibitors that induce apoptosis and neurotoxic effects at certain concentrations on a hippocampal cell line (Chung et al., 2012; Post et al., 2000). Deprenyl increases neuronal cell viability and reduces ethanol-induced apoptosis by reducing MAO B and transforming growth factor-beta-inducible early gene 2 (TIEG2) levels (Lu et al., 2008). It also alters the gene expression of heme-oxygenase in neuroblastoma cells by changing dopamine levels (Rieder et al., 2004).

1.4.1.3 Anti-Inflammatory Effects of MAO Inhibitors

MAO inhibitors have demonstrated anti-inflammatory properties in various cells. Glial activation and subsequent neuroinflammation in neurodegenerative diseases result in the secretion of various cytokines such as IL-1β and TNFα exhibited by a LPS-induced rat mixed glial cell culture model (Bielecka et al., 2010). Moclobemide over a wide concentration range diminished pro-inflammatory cytokines and mRNA in the supernatant of treated cells and the translocation of the p65 subunit of NF-κB into cellular nuclei (Bielecka et al., 2010). TNFα and IL-6 mRNA are also upregulated in the hippocampus of patients with Parkinson’s disease and MAO inhibitors have shown to induce the release of anti-inflammatory cytokines to counteract this effect (Nagatsu and Sawada, 2006). MS, an inflammatory demyelinating disease, is modeled by experimental autoimmune encephalomyelitis (EAE) in mice. Disease severity in the chronic phase was reduced, onset delayed and exploratory behavior improved with treatment of phenelzine. Serotonin levels were also normalized demonstrating the potential use of therapeutic MAO inhibitors such as phenelzine for MS treatment (Musgrave et al., 2011). Microglia and macrophage activation induced by LPS leads to axonal degeneration in a primary rat microglial cell model. The use of safinamide, a MAO B inhibitor, suppresses microglial superoxide production, enhances glutathione, an anti-oxidant, and protects axons from degeneration (Morsali et al., 2013).
Major depressive states can activate the inflammatory response system thus increasing pro-inflammatory cytokines, positive acute phase proteins and PGE2. A model of LPS stimulation of whole blood induced the release of IL-6, IL-8, TNFα, IL-10 and IL-1 and was attenuated with moclobemide co-treatment (Lin et al., 2000). Similar results were found with monocyte stimulation of pro-inflammatory cytokine release. In psoriasis, a chronic inflammatory skin disease, lesions have been decreased with MAO inhibitors and E. coli – endotoxin induced mortality of mice is inhibited by amine oxidase inhibition (Davis et al., 1962). PGE2 synthesis was inhibited in patients with rheumatoid arthritis with MAO inhibitors resulting in remission (Lieb et al., 1983).

Mouse models of UC where mice have been challenged with LPS have been improved with LJP 1207, an amine oxidase inhibitor, reducing mortality, loss of body weight, ulceration scores, colonic TNFα and IL-6 levels, inflammation and swelling (Salter-Cid et al., 2005). A case study demonstrated that phenelzine enhances gut mucosal barrier function and decreases luminal antigen permeability in a CD patient (Kast, 1998). Daily administration of the drug improved the patient’s depression, abdominal discomfort, bowel movements and joint pain and removal of treatment resulted in relapse of CD (Kast, 1998).

Topical application of a MAO inhibitor also successfully counteracted LPS-induced elevation of H$_2$O$_2$, MAO B and TNFα expression in a rat periodontal disease model both in vivo and in vitro (Ekuni et al., 2009). Disease-associated characteristics such as migration and proliferation of junctional epithelium, alveolar bone loss and systemic H$_2$O$_2$ levels were mitigated with the MAO inhibitor, introducing one of the first examples of MAO inhibitors on improvement of epithelial barrier integrity, a key target in mucosal inflammatory diseases.

With the evidence presented thus far, MAO inhibitors may provide a clinical benefit in treatment of acute and chronic inflammatory diseases.

### 1.4.2 Novel MAO B Inhibitors

Similar to MAO A inhibitors, MAO B inhibitors are not free of side effects. Common side effects such as insomnia, dizziness, headaches and increase in dopamine levels are caused because the inhibitors are able to transit into the brain. It also has established drug interactions since it crosses the blood brain barrier (BBB) and results in severe outcomes. For example, serotonin syndrome occurs when serotonin levels are elevated in the brain
leading to excessive nerve cell activity and causing potentially dangerous symptoms such as seizures, irregular heartbeat and high fever. This significantly limits the clinical utility of existing MAO B inhibitors. Currently, the Putnins research group is collaborating with the Centre for Drug Research and Development (CDRD) to develop novel MAO B inhibitors that no longer cross the BBB thus have less adverse effects while maintaining its activity in peripheral tissues.

1.4.2.1 Characteristics of Novel MAO B Inhibitors

Passage of drugs across the BBB is generally restricted to non-polar or hydrophobic drugs. Therefore, appropriate alteration of polar functionality is able to suppress novel MAO B inhibitor entry into the brain. Dr. David Grierson from the Faculty of Pharmaceutical Sciences at the University of British Columbia has designed novel, potent, reversible and selective MAO B inhibitors with reduced transit across the BBB. Three zones of deprenyl are altered making way for the synthesis of novel deprenyl analogues (Figure 8).

![Figure 8. Structure of deprenyl](image)

The structure of deprenyl and the three zones that are altered to synthesize novel MAO B inhibitors that do not cross the BBB yet maintain its inhibitory activity. Reprinted by permission from CDRD.

A biochemical assay was utilized to screen the novel MAO B inhibitors for MAO B activity and isoform selectivity over MAO A. This assay functioned as a screen and allows us to select lead novel MAO B inhibitors based on the following criteria: IC$_{50}$ of less than 300 nanomolar, selectivity of MAO B inhibition over MAO A of 100-fold and reduced transit across the BBB using an in vitro BBB model. To date, 50 compounds have been synthesized and a patent (WO/2015/027324) was issued in March 2015 (Putnins et al., 2015).
There are currently four lead novel MAO B inhibitors that were selected and are designated as compounds A, B, C and D (Figure 9). These novel MAO B inhibitors exhibit distinct selectivity of MAO B inhibition over MAO A and have IC\textsubscript{50}’s of MAO B inhibition of 270 nM (Compound A), 200 nM (Compound B), 210 nM (Compound C) and 300 nM (Compound D) (Figure 9). The novel MAO B inhibitors also demonstrated reduced permeability across the BBB and are reversible compounds, contrary to the irreversible parent MAO B inhibitor deprenyl. Compounds A, B, C and D are derived from alterations in zones 1 and 2 of deprenyl while compound D is derived from alterations of all three zones of the parent compound (Figure 8).

**Figure 9. Novel MAO B inhibitor isoform selectivity and IC\textsubscript{50}’s**

Compounds A, B, C and D exhibit specific selectivity of MAO B inhibition over MAO A and IC\textsubscript{50}’s of MAO B inhibition for the four lead novel MAO B inhibitors are 300 nM or lower. (Reprinted by permission from C.Kim of CDRD).
1.5 Rationale, Objectives and Hypothesis of this Study

**Rationale:** MAO B is induced in epithelial cells of a rat periodontal disease model and topical application of a known MAO inhibitor dramatically reduced characteristics associated with chronic inflammation (Ekuni et al., 2009). However, the therapeutic potential of MAO B inhibitors on GI mucosal tissues has not been extensively examined. In IBDs, intestinal epithelial and endothelial cells are important cell populations as they are situated at the forefront of inflammatory injury (Medzhitov and Janeway, 2002). These cells respond to LPS and secrete pro-inflammatory cytokines such as IL-8, IL-6, TNFα and IL-1β, similar to the cytokine profile established in IBDs (Cromer et al., 2011). Currently available MAO inhibitors have adverse side effects due in part to their ability to cross the BBB, thus novel selective MAO B inhibitors that have reduced transit into the brain will be tested to determine their impact on mediators of inflammation.

**Global Objective:** We will examine in intestinal epithelial and endothelial cell culture the potential of novel MAO B inhibitors (compounds A, B, C and D) to reduce LPS-induced cytokine secretion and gene expression *in vitro*.

**Specific Aims:**

1) Examine MAO B and MAO A protein expression in intestinal epithelial and endothelial cells and the effect of novel MAO B inhibitors and deprenyl on cell viability, cytotoxicity and induction of apoptosis.

2) Examine the effect of deprenyl and novel MAO B inhibitors on LPS-induced pro-inflammatory cytokine secretion in conditioned media of intestinal epithelial and endothelial cells using a multiplex assay.

3) Examine the effect of deprenyl and novel MAO B inhibitors on LPS-induced pro-inflammatory cytokine gene expression of intestinal epithelial and endothelial cells using quantitative real-time polymerase chain reaction (qRT-PCR).

**Hypothesis:** Novel MAO B inhibitors and deprenyl will not decrease viability, nor increase cytotoxicity or apoptosis and will reduce LPS-induced pro-inflammatory cytokine secretion and gene expression in intestinal epithelial and endothelial cells.
Chapter 2: Novel MAO B Inhibitors Reduce Pro-Inflammatory Cytokine Secretion and Gene Expression Induced by LPS Challenge in Intestinal Epithelial and Endothelial Cells

2.1 Materials and Methods

2.1.1 Materials

25cm² (T25), 75cm² (T75), 175cm² (T175) tissue culture flasks, polypropylene centrifuge tubes, 96-well and 12-well cell culture plates and MCDB 131 media were from Sarstedt (Numbrecht, DK) and Corning Incorporated Life Sciences (Lowell, MA). Microcentrifuge tubes, white polystyrene reservoirs and 96-well flat clear bottom black polystyrene TC-treated microplates were from Van Waters Rogers International (Radnor, PA). Endothelial Cell Growth Supplement, Blok-FL Blocking Agent and Immobilon-FL Transfer Membrane were from Millipore (Billerica, MA). RIPA Lysis Buffer was from Santa-Cruz Biotechnology (Dallas, TX). Primary antibodies of rabbit anti-MAO A (cat no. ab126751), rabbit anti-MAO B (cat no. ab 125010) and mouse anti-actin (cat no. ab 3280) were purchased from Abcam (Toronto, ON). Secondary antibodies of donkey anti-rabbit IgG IR Dye 800CW (cat no. 926-32213) and donkey anti-mouse IgG IR Dye 680RD (cat no. 926-68072) were purchased from LI-COR Biosciences (Lincoln, NE). Digitonin, ionomycin, staurosporine, TNFα, heparin sodium salt, fibronectin from bovine plasma, lipopolysaccharides from Escherichia coli 055:B5 and deprenyl (cat no. M003) were purchased from Sigma (Oakville, ON).

The ApoTox-Glo™ Triplex Assay and CellTiter-Blue® Cell Viability Assay were from Promega Corporation (Madison, WI). The V-PLEX Human Proinflammatory Panel I (4-Plex) Kit and Human ProInflammatory 7-Plex Ultra-Sensitive Plate were from Meso Scale Discovery (MSD) (Rockville, MD). The RNeasy Mini Kit, QIAshredder homogenizer and RNase-Free DNase Set were from Qiagen (Standford, CA). The Pierce® BCA Protein Assay Kit, High Capacity cDNA Reverse Transcription Kit, custom Taqman® Gene Expression assays for human TNFα (Hs99999043_m1), IL-8 (Hs00174103_m1), IL-6 (Hs00985639_m1), MAO-B (Hs01106246_m1), MAO-A (Hs00165140_m1), GAPDH (Hs02758991_g1), TaqMan Fast Advanced Master Mix, MicroAmp® Fast 96-well reaction
plates and MicroAmp™ optical adhesive film were from Applied Biosystems (ABI) by Life Technologies (Grand Island, NY).

Caco-2 cells (ATCC® HTB-37) at passage 26 and human embryonic kidney 293 cells (HEK 293 ATCC® CRL-1573) at passage 43 were generously supplied by Dr. Pauline So (Target Validation division, CDRD). Human intestinal microvascular endothelial cells (HIMEC), derived from normal sigmoid tissue of a diverticulitis 48-year old female at passage 4, were generously donated by Dr. John Hegarty (Department of Surgery, Penn State University College of Medicine). Dulbecco’s Modified Eagle Medium (DMEM), fetal bovine serum (FBS), 0.05% Trypsin-EDTA (TE) (1X), GlutaMAX™ Supplement (100X), Penicillin-Streptomycin (10,000 U/mL), phosphate buffered saline (PBS), pH 7.4 and Antibiotic-Antimycotic (100X) were from Gibco (Grand Island, NY).

2.1.2 Epithelial and Endothelial Cell Culture Experiments

Caco-2 cells between Passage 28-42 were cultured in DMEM containing 10% FBS, 1% GlutaMAX™ and 1% Penicillin-Streptomycin. Caco-2 cells were cultured on T75 flasks and were maintained at 37°C in a humidified atmosphere of 95% air 5% CO₂. To detach the confluent cells from tissue culture flasks, they were washed twice with 5 mL of PBS and treated with 3 mL of 0.05% TE for 5 minutes. Cells were resuspended in 15 mL of fresh medium. 1 mL of this suspension was analyzed by a vi-CELL® Series Cell Viability Analyzer (Beckman Coulter, Inc, Mississauga, ON) where viable cells were counted and concentration of cell suspension was calculated. The remaining cells were pelleted by centrifuging for 5 minutes at 1300 rpm. The culture medium was aspirated and resuspended in fresh medium at a concentration of 10⁶ cells/mL. 10⁵ cells were seeded onto a new T75 flask for further cultivation or plated for the ApoTox Glo Triplex Assay, V-PLEX Human Proinflammatory Panel I (4-Plex) Kit or qRT-PCR at the appropriate densities.

HIMEC cells between Passage 8-12 were cultured in MCDB 131 containing 20% FBS, 1% GlutaMAX™, 1% Antibiotic-Antimycotic and 3.8% heparin sodium salt (2.5 mg/mL). 50 μg/mL of freshly thawed endothelial cell growth supplement was added to the working media. HIMEC cells were cultured on 1.25 μg/cm² fibronectin-coated T25 flasks and were maintained at 37°C in a humidified atmosphere of 95% air 5% CO₂. To detach
confluent cells, they were washed once with 5 mL of PBS and treated with 3 mL of 0.05% TE (1X) for 1 minute. Cells were harvested into 10 mL of trypsin-neutralizing solution (5% FBS in PBS) and resuspended. 10 μL of this suspension was added to 10μL of Trypan Blue solution where viable cells were counted using a hemocytometer two times to ensure accurate cell counts. The total number of cells and concentration of cell suspension were calculated. The remaining cells were pelleted by centrifuging for 5 minutes at 1300 rpm. The trypsin-neutralizing solution was aspirated and the cell pellet was resuspended in fresh medium. Cells were seeded into two or more fibronectin-coated T75 flasks with at least 2x10^4 cells/mL densities. Once confluent, HIMEC cells were harvested and plated for the ApoTox Glo Triplex Assay, V-PLEX Human Proinflammatory Panel I (4-Plex) Kit or qRT-PCR at the appropriate densities.

2.1.3 Analysis of Protein Expression

MAO A and B protein expression in Caco-2 and HIMEC cells were assessed by Western Blot as described by Guo et al. (2013). Caco-2 and HIMEC cells were plated on T75 flasks in 10%- and 20% FBS-containing media, respectively and grown until confluence. Cells were washed twice with warm PBS and harvested using 0.05% TE into microcentrifuge tubes, centrifuged, washed 3 times with ice-cold PBS and lysed with 50 to 200 μL of RIPA Lysis Buffer with the addition of protease and phosphatase inhibitors. After thirty minutes of incubation on ice, the tubes were spun at 4°C for 15 minutes at 13000 rpm. The supernatant was collected and protein concentration was determined using the Pierce® BCA Protein Assay Kit. Laemmli gel loading buffer [5mL 0.5 M Tris pH 6.8, 0.8 g sodium dodecyl sulfate (SDS), 4mL Glycerol, 0.005 g Bromophenolblue, 800 μL β-Mercaptoethanol and H₂O] was added to the lysate containing 35 μg of protein and boiled for 5 minutes. Proteins were separated on a 10% SDS/polyacrylamide gel electrophoresis gel and transferred to an Immobilon-FL Transfer Membrane. The membrane was incubated in blocking buffer (1:1 of FL-blok blocking buffer to PBS) for 30 minutes then incubated in diluted primary antibody (either Rabbit anti-MAO A or anti-MAO B at 1:1000) overnight at 4°C. After three washes in TBS-0.1% Tween buffer, the membrane was incubated in donkey anti-rabbit IgG IR Dye 800CW secondary antibody (1:15000) for one hour and scanned using the LI-COR Odyssey
Scanning Platform (LI-COR, Inc). The membrane was then washed in TBS-0.1% Tween buffer and incubated with the primary Mouse anti-Actin antibody overnight. After three washes in TBS-0.1% Tween buffer, the membrane was incubated in donkey anti-mouse IgG IR Dye 680RD secondary antibody (1:15000) for one hour and scanned again. Fluorescence intensities of bands were determined using the Image Studio Software (LI-COR, Inc).

2.1.4 Analysis of Cell Viability, Cytotoxicity and Apoptosis

Caco-2 cells were seeded in a 96-well plate at 4.9 x 10^4 cells/cm^2 in DMEM with 10% FBS media while HIMEC cells were seeded in 1.25 µg/cm^2 fibronectin-coated 96-well plates at 7.0 x 10^4 cells/cm^2 in MCDB 131 with 20% FBS media and allowed to adhere and grow on the plate for 24 hours at 37°C and 5% CO_2. Cells were maintained in their respective media (with either 10% or 20% FBS) throughout treatments. Cells were treated in quadruplicates for 24 hours with 10, 20 or 40 µM of novel MAO B inhibitors or deprenyl, 6 hours with 10 µM of staurosporine or 100 µM of ionomycin and 15 minutes with 24 µM of digitonin. These same treatments were added to non-cell wells to serve as medium-only controls for background fluorescence or luminescence readings. Medium with sterile water was added to replicate wells to serve as vehicle cell controls. After 24-hour cell treatments were complete, a fluorogenic viability and cytotoxicity reagent was prepared by adding 10 µL of GF-AFC and bis-AAF-R110 substrates in 2 mL of assay buffer provided by the ApoTox-Glo™ Triplex Assay Kit as previously described (Niles et al., 2007). 20 µL of the reagent was delivered to all wells, mixed by orbital shaking for 30 seconds at 500 rpm and returned to a 37°C incubator for 30 minutes of incubation. The resulting fluorescence was measured in the AFC (400_{Ex} 505_{Em}) and R110 (485_{Ex} 520_{Em}) channels using a SpectraMax M5 Microplate Reader (Molecular Devices LLC) and analyzed using the Soft Max Pro 5 Acquisition and Analysis Software (Molecular Devices LLC). Caspase-Glo® 3/7 Reagent, another component of the ApoTox-Glo™ Triplex Assay Kit, was prepared by adding the Caspase-Glo® 3/7 buffer to the luminogenic substrate (containing the DEVD sequence). 100 µL was added to all wells, mixed by orbital shaking and incubated for 30 minutes at room temperature in the dark. Luminescence was measured using the SpectraMax M5 and analyzed using the Soft Max Pro 5 Acquisition and Analysis Software.
2.1.5 Analysis of Pro-Inflammatory Cytokine Protein Secretion

Caco-2 cells were seeded in a 96-well plate at 1.4 x 10^5 cells/cm^2 in DMEM with 10% FBS media while HIMEC cells were seeded in a 1.25 µg/cm^2 fibronectin-coated 96-well plate at 8.4 x 10^4 cells/cm^2 in MCDB 131 with 20% FBS media and allowed to adhere and grow on the plate for 24 hours at 37°C and 5% CO₂. On Day 1, wells were replaced with fresh DMEM media with 10% FBS for Caco-2 cells or MCDB 131 media with 20% FBS for HIMEC. On Day 2, wells were replaced with fresh MCDB 131 media or DMEM media with 2.5% FBS. Cells were maintained in their respective media (with 2.5% FBS) throughout treatments. Depending on the experiment, cells were treated in biological triplicates for 1, 3, 4, 12 or 24 hours with 10, 20 or 40 µM of the novel MAO B inhibitors or deprenyl alone or with 1 µg/mL LPS with or without deprenyl or the novel MAO B inhibitors at .1, 1, 5, 10, 20 or 40 µM. After treatment incubations were complete, the cell supernatant was collected and the level of cytokine secretion in the conditioned media was assayed using either a 7-spot or 4-spot electrochemiluminescent immunoassay from MSD as described previously (Cho et al., 2014; Fiorentino et al., 2014; Knudsen et al., 2014; Lin et al., 2013). 25 µL of the collected cell culture supernatant for each treatment and duplicates of serial-diluted calibrators provided by MSD was dispensed in either a Human ProInflammatory 7-Plex Ultra-Sensitive Plate or V-PLEX Human Proinflammatory Panel 1 (4-Plex) Plate and incubated at room temperature on a shaking platform for 2 hours. The MSD cytokine standards were serial diluted 1/4 in either DMEM media for Caco-2 cells or MCDB 131 media for HIMEC cells to produce duplicate 8-standard concentrations. Each cytokine at the diluted concentrations produced standard curves with different detection ranges since each cytokine stock concentration varied (data not shown). Preparing the standard concentrations with the stock cytokine in either Reagent 2 from the MSD kit or in media did not produce different standard curves (data not shown). The working electrodes on the 7-plex plate were coated with capture antibodies for IL-1β, IL-12p70, IFN-γ, IL-6, IL-8, IL-10 and TNF-α while the 4-plex plate working electrodes were coated with capture antibodies for IL-1β, IL-6, IL-8 and TNF-α. The plate was washed 3 times with wash buffer and 25 µL of detection antibody diluted in antibody diluent (1X) was added and incubated for 2 hours at room temperature on a shaking
platform. The plate was washed 3 times with wash buffer, 150 µL of 2X Read Buffer T was added to each well and electrochemiluminescence was measured using the Sector Imager 2500A (MSD, Gaithusburg, MD). Samples were analyzed in biological triplicates and standards were analyzed in technical duplicates with a maximum tolerated coefficient of variation of 20%.

2.1.6 Analysis of Pro-Inflammatory Cytokine Gene Expression

Caco-2 cells were seeded in a 12-well plate at 5.0 x 10⁴/cm² in DMEM with 10% FBS media while HIMEC cells were seeded in a 1.25 µg/cm² fibronectin-coated 12-well plate at 2.6 x 10⁴/cm² in MCDB 131 with 20% FBS media and allowed to adhere and grow on the plate for 24 hours at 37°C and 5% CO₂. On Day 1, wells were replaced with fresh DMEM media with 10% FBS for Caco-2 cells or MCDB 131 media with 20% FBS for HIMEC. On Day 2, wells were replaced with fresh MCDB 131 media or DMEM media with 2.5% FBS. Cells were maintained in their respective media (with 2.5% FBS) throughout treatments. Cells were treated in three independent experiments for 4 or 12 hours with 20 µM of novel MAO B inhibitors or deprenyl alone or with 1 µg/mL LPS with or without deprenyl or novel MAO B inhibitors at 20 µM. For each time point, cell lysates were collected, homogenized with the QIAshredder homogenizer and total RNA was isolated using the RNeasy Mini Kit (Qiagen) including an on-column DNase digest with the RNase-Free DNase Set following the manufacturer’s instructions. RNA was eluted in nuclease-free water (Ambion) and the nucleic acid purity and concentration were assessed using the NanoDrop 1000 Spectrophotometer (ThermoScientific) from A₂₆₀, A₂₈₀ and A₂₃₀ measurements.

qRT-PCR was used to evaluate changes in gene expression. cDNA synthesis was performed for each biological triplicate. For each 20 µL reverse transcription reaction, depending on the available RNA concentration, 200 ng to 2 µg of total RNA (in 10 µL water) was mixed with 10 µL 2X RT master mix (2µL RT buffer, 0.8 µL dNTP Mix (100 mM), 2 µL 10X RT Random Primers, 1 µL MultiScribe Reverse Transcriptase and 4.2 µL Nuclease-free H₂O) provided in the High Capacity cDNA Reverse Transcription Kit (ABI). Reverse transcription was performed for 10 min at 25°C and 120 min at 37°C for primer annealing
and extension and terminated by incubating for 5 min at 85°C on the Mastercycler gradient thermal cycler (Eppendorf).

The selection of pre-designed target primers and probes of interest was determined by the cytokine secretion results obtained from the MSD V-PLEX Human Pro-Inflammatory Panel I (4-Plex) experiments. Primer and probe sequences were selected using the Assay Search Tool (ABI) and were specific to the target gene sequences. The selected Taqman Gene Expression Assays ending in “_m1” for IL-6, IL-8 and TNFα indicated the assay spanned an exon junction and did not detect genomic DNA and were labeled with the fluorescent reporter dye FAM. MAO A and B primer and probe non-specific cross-reactivity with gene targets was disproved using BLASTn analyses of 40 nucleotides spanning the left and right sides of the indicated assay location. These analyses revealed no overlap of MAO B sequence with MAO A and only 84% identities of a 64% query cover of the MAO A sequence was identical to MAO B. The pre-designed assay for the housekeeping gene, GAPDH, ended in “_g1” indicating the assay may have detected genomic DNA since the probe and primer could be within a single exon and was labeled with the fluorescent reporter dye VIC. GAPDH is a housekeeping gene that has been extensively used for both Caco-2 and HIMEC cells previously (Nishitani et al., 2013; Piana et al., 2008; Vreeburg et al., 2011).

In general, amplicons were between 65 and 100 nucleotides. TaqMan Fast Advanced Master Mix (AmpliTaq Fast DNA Polymerase, Uracil-N glycosylase, dNTPs with dUTP, passive reference ROX dye and optimized buffer components) was used for all reactions along with the necessary TaqMan Gene Expression Assay for each gene of interest. The TaqMan Gene Expression Assay for GAPDH was included in each PCR reaction as an endogenous control. Parallel reactions for each cDNA sample with the original RNA or non-template control (NTC) samples were run to assess the degree of any contaminating genomic DNA. Four preliminary TaqMan assays for all genes of interest and GAPDH were run to assess sample integrity and PCR efficiencies between 90 to 110% were confirmed between all primer/probe sets for both cell types.

TaqMan PCR assays for each gene target were performed in technical triplicate for each biological triplicate cDNA or NTC sample in MicroAmp® Fast 96-well reaction plates sealed with MicroAmp™ optical adhesive films (ABI). For each 20 µL TaqMan reaction, 5
µL of 10 ng cDNA or NTC was mixed with 10 µL TaqMan Fast Advanced Master Mix (2X), 1 µL TaqMan Gene Expression Assay (20X) for the gene of interest, 1 µL TaqMan Gene Expression Assay (20X) for GAPDH and 3 µL of nuclease-free water. qRT-PCR was run on the 7900HT Fast Real-Time PCR System (ABI). The thermal cycling profile were as follows: initiation required for optimal UNG activity (50°C for 2 minutes), activation of the AmpliTaq Fast DNA Polymerase (95°C for 20 seconds), followed by 50 cycles of denaturation (95°C for 1 second) and annealing/extension (60°C for 20 seconds). All TaqMan PCR data were captured using Sequence Detector Software (SDS version 2.3; ABI) and analyzed using the Relative Quantitation (RQ) manager software (ABI). For every sample, a threshold cycle (Ct) value was calculated for each gene of interest and corrected using the Ct value obtained from the endogenous gene yielding a ΔCt value. Relative quantification was calculated according to the ΔΔCt method as described previously (Livak and Schmittgen, 2001). ΔΔCt values for each sample were compared either to ΔΔCt values of the control or LPS-treated samples to produce fold-change values using the equation $2^{-\Delta\Delta C_t}$. The mean ± standard error of the mean (SEM) of each treatment, relative to control- or LPS-treated samples, is presented in graphs of Chapter 2.2.4. Therefore, control or LPS-treated samples are depicted as having a 1.0 fold-change as indicated.

2.1.7 Statistical Analysis

Data analysis was performed using SPSS Statistics Software (IBM) and graphed using Prism (GraphPad). All data are expressed as mean values with their standard errors or standard deviations (Std Dev) as indicated in the figure legends. Independent sample groups were assessed for homogeneity of variances by Levene’s Test. When homogeneous variances were confirmed, the data were analyzed by one-way ANOVA followed by pairwise comparisons of treatment means using the Tukey Post-Hoc test. When unequal variances were detected, the data were analyzed using the non-parametric Kruskal-Wallis test with the Dunn’s post-hoc test or the Student’s t-test with a Bonferroni correction. Differences were considered significant when $p<0.05$. 

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2.2 Results

2.2.1 MAO A and B Expression in Selected Cell Lines

To validate our choice of cell line, MAO A and B protein expression was determined in Caco-2 and HIMEC cell lysate samples via Western blot analysis using a rabbit anti-MAO A or rabbit anti-MAO B primary antibody. MAO B (~59 kDa) was observed in both cell lysates but not MAO A (~60 kDa) (Figure 10). Positive control HepG2 cell lysates stained with both anti-MAO A and -B antibodies, while the recombinant MAO A protein stained with anti-MAO A antibody. Since Caco-2 and HIMEC cells were confirmed positive for MAO B and not MAO A protein expression, they were selected as cell lines to use for the analysis of novel MAO B inhibitors in vitro.

![Western Blot Image]

**Figure 10. Caco-2 and HIMEC cells express MAO B but not MAO A protein**
Protein expression of MAO A and B in Caco-2 and HIMEC cells were determined by Western Blot analysis. A representative blot of two experiments is shown. β-Actin (red) was used as a loading control and the recombinant MAO A protein and HepG2 cell lysates were used as positive controls. Blot was stained with anti-MAO B antibody (green; lanes 1-3) and anti-MAO A antibody (green; lanes 5-9). Lane 4 shows the molecular weight marker.
To determine if gene expression coincided with protein expression, MAO A and B mRNA levels were assessed in total RNA isolated from Caco-2 and HIMEC cells via qRT-PCR analysis using Taqman gene expression assays. Unlike the Western Blot results, MAO A and B gene expression did not parallel protein expression in both Caco-2 and HIMEC cells. In Caco-2 cells, MAO A mRNA levels in vehicle-treated control cells were significantly higher relative to MAO B at 4 hours, indicative of a lower ΔCt value for MAO A (9.3) compared to MAO B (10.7) and at 12 hours with a lower ΔCt for MAO A (10.1) compared to MAO B (11.0) (Table 1A). In HIMEC cells, MAO A mRNA levels were detected at 4 and 12 hours while MAO B was not detected (Table 1B). Statistical evaluation could not be completed for the relative expression of MAO A and B mRNA in HIMEC cells as MAO B experimental analysis was performed with one biological replicate. However, MAO B mRNA levels were consistently below the limit of detection in HIMEC cells in three pilot experiments (data not shown).

Table 1. Baseline MAO A and B Gene Expression in Caco-2 and HIMEC Cells

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<th>Caco-2 Cells</th>
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<th>HIMEC Cells</th>
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<td></td>
<td>4h</td>
<td>12h</td>
<td>4h</td>
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<tr>
<td>MAO A</td>
<td>9.3 ± 0.29</td>
<td>10.1 ± 0.32</td>
<td>13.44 ± 0.13</td>
<td>13.06 ± 0.42</td>
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<tr>
<td>MAO B</td>
<td>10.7 ± 0.29**</td>
<td>11.0 ± 0.25*</td>
<td>ND</td>
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</table>

Caco-2 (A) and HIMEC (B) cells were cultured on 12-well plates in 10% and 20% FBS media, respectively. On Day 2 after seeding, serum concentration was reduced to 2.5%. On Day 4, total RNA was harvested after 4 and 12 hours of water (vehicle control) treatment. Using reverse transcriptase, cDNA was synthesized from 2 µg total RNA and used as template for qRT-PCR reactions using primers and probes for MAO A or MAO B and GAPDH. Each reaction contained 10 ng cDNA derived from total RNA. Three biological replicates of each reaction were performed with technical triplicates of each. Results are expressed as mean ΔCt ± Std Dev, where lower ΔCt values indicate higher gene expression levels. *p<.05, **p<.01. ND, not detectable.
2.2.2 (-)-Deprenyl and Novel MAO B Inhibitors Do Not Reduce Viability nor Increase Cytotoxicity or Apoptosis of Caco-2 and HIMEC Cells

To assess if the novel compounds or deprenyl at 10, 20 or 40 µM affected Caco-2 or HIMEC cell viability, cytotoxicity or apoptosis, these three characteristics were measured by the Apotox-Glo Triplex Assay. The positive controls of 100 µM ionomycin, 24 µM digitonin and 10 µM staurosporine induced cytotoxicity or apoptosis, respectively, for both Caco-2 (Figure 11 ACE) and HIMEC cells (Figure 11 BDF), as indicated by decreased relative fluorescence units (RFU) associated with viability and increased RFU or RLU (relative luminescence units) associated with cytotoxicity or apoptosis. Ionomycin decreased Caco-2 cell viability (14.03 RFU) compared to vehicle control (60.4 RFU) (Figure 11A). In HIMEC cells, ionomycin reduced viability (26.7 RFU) compared to vehicle control (180 RFU) (Figure 11B). Ionomycin induced cytotoxicity in Caco-2 cells (178 RFU) significantly over vehicle control (5.68 RFU) (Figure 11C). Digitonin induced cytotoxicity in HIMEC cells (257 RFU) over vehicle control cells (50.2 RFU) (Figure 11D). Staurosporine induced apoptosis in Caco-2 cells (664 RLU) compared to vehicle control (250 RLU) (Figure 11E). In HIMECs, staurosporine induced apoptosis (3930 RLU) compared to vehicle control (3230 RLU) (Figure 11F).

Deprenyl and the novel compounds at 10, 20 and 40 µM concentrations did not decrease Caco-2 or HIMEC cell viability as these treatments did not significantly decrease RFU associated with cell viability compared to controls (Figure 11A and B). Accordingly, deprenyl and the novel compounds did not increase Caco-2 or HIMEC cell cytotoxicity (Figure 11 C and D). Deprenyl and the novel compounds did not increase Caco-2 or HIMEC cell apoptosis (Figure 11 E and F). Compound D at 20 µM (81.3 RLU) and 40 µM (36.5 RLU) had significantly lower luminescence values associated with apoptosis compared to vehicle control (250 RLU) (Figure 11E). Compound D decreased apoptosis in a concentration-dependent manner when compared to vehicle control cells, indicating that this compound may have anti-apoptotic properties.
Figure 11. Novel MAO B inhibitors and deprenyl do not decrease viability nor increase cytotoxicity or apoptosis in Caco-2 and HIMEC cells

Caco-2 (left panel) and HIMEC (right panel) cells were cultured on a 96-well plate in media containing 10% and 20% FBS, respectively. Cells were treated for 24 h with 10, 20 or 40 µM of deprenyl, compound A, B, C or D. Analysis of either RFU associated with (A, B) viability and (C, D) cytotoxicity or RLU associated with (E, F) apoptosis was determined. Significant differences were found between vehicle control (VC) and positive controls ionomycin (Ion), digitonin (Dig) and staurosporine (Sta). n=3 (controls), n=4 (treatments). Results are expressed as mean ± Std dev. ***p<.001
2.2.3 Regulation of LPS-Induced Pro-Inflammatory Cytokine Secretion by Novel MAO B Inhibitors in Caco-2 and HIMEC Cells

Human epithelial and endothelial cell lines were initially screened for baseline expression of IL-1β, IL-10, IFN-γ, IL-12p70, TNFα, IL-6 and IL-8 secretion and compared to the relative expression in LPS-stimulated cultures. Standard curves were used to determine absolute concentrations of each cytokine.

There was a statistically significant increase in IL-10, IL-12p70, IL-6 and IL-8 in Caco-2 cells treated with 1 µg/mL LPS (Figure 12). At 24 hours of LPS treatment, IL-10 (0.70 pg/mL) was secreted from Caco-2 cells (Figure 12B). IL-12p70 was secreted at 4 hours (0.86 pg/mL), 12 hours (0.72 pg/mL) and 24 hours (0.87 pg/mL) with LPS treatment (Figure 12D). Basal IL-6 secretion increased with LPS treatment for 4 hours (0 pg/mL to 0.54 pg/mL), 12 hours (0.08 pg/mL to 1.42 pg/mL) and 24 hours (0.25 pg/mL to 1.22 pg/mL) (Figure 12F). Out of the four cytokines that were significantly increased over basal secretion levels, IL-8 was secreted from Caco-2 cells in the most abundance. Basal levels of IL-8 increased with LPS treatment at 4 hours (1.78 pg/mL to 96.2 pg/mL), 12 hours (5.15 pg/mL to 193 pg/mL) and 24 hours (10.2 pg/mL to 200 pg/mL) (Figure 12G).
Figure 12. Increased pro-inflammatory cytokine secretion in Caco-2 cells treated with LPS for 4, 12 and 24 hours

Caco-2 cells were cultured on a 96-well plate in DMEM medium containing 10% FBS. On day 5 after seeding, wells were replaced with fresh media containing 2.5% FBS. Cells were treated for 4, 12 or 24 h on day 6 or 7 with 1 µg/mL LPS. Supernatants of the treated cells were analyzed for pro- and anti-inflammatory cytokines using the Human Proinflammatory 7-Plex Ultra-Sensitive Kit. Absolute concentrations of (A) IL-1β, (B) IL-10, (C) IFNγ, (D) IL-12p70, (E) TNFα, (F) IL-6 and (G) IL-8 induced with 1 µg/mL LPS are shown. Results are expressed as mean ± Std dev (n=3). *p<.05, **p<.01 vs VC (Vehicle control).
There was a statistically significant increase in all cytokines analyzed in the 7-plex Ultra-Sensitive Kit in HIMEC cells treated with 1 µg/mL LPS (Figure 13). HIMEC cells treated with 1 µg/mL LPS for 3 hours secreted IL-1β (0.77 pg/mL, Figure 13A), IL-10 (1.77 pg/mL, Figure 13B), IFN-γ (5.33 pg/mL, Figure 13C) and IL-12p70 (3.99 pg/mL, Figure 13D) over basal levels (0 pg/mL). Out of the seven cytokines that were significantly increased, TNFα, IL-6 and IL-8 were secreted at higher concentrations and were induced with both 0.1 and 1 µg/mL LPS treatment in HIMEC cells, demonstrating a concentration-dependent increase in these three cytokines with endotoxin treatment. HIMECs treated with 0.1 µg/mL LPS secreted TNFα at 3 hours (19.0 pg/mL) (Figure 13E). HIMECs treated with 1 µg/ml LPS secreted TNFα at 1 hour (4.77 pg/mL) and 3 hours (84.1 pg/mL) over basal levels (0 pg/mL) (Figure 13E). Basal levels of IL-6 at 1 hour (0 pg/mL) increased with 0.1 µg/mL LPS (0.47 pg/mL) and 1 µg/mL LPS (6.07 pg/mL) (Figure 13F). The basal level of IL-6 at 3 hours (0.98 pg/mL) increased with 0.1 µg/mL LPS (47.7 pg/mL) and 1 µg/mL LPS (189 pg/mL) (Figure 13F). Lastly, IL-8 levels at 1 hour (101 pg/mL) increased with 0.1 µg/mL LPS (226 pg/mL) and 1 µg/mL LPS (493 pg/mL) (Figure 13G). At 3 hours, the IL-8 basal level (492 pg/mL) increased with 0.1 µg/mL LPS (2110 pg/mL) and 1 µg/mL LPS (2670 pg/mL) (Figure 13G).

This preliminary screen demonstrated that mainly IL-8, IL-6 and TNFα were secreted from HIMEC and Caco-2 cells with LPS treatment, which led to the decision of focusing on these particular cytokines in subsequent assays. In the assay kit selected, IL-1β was present as well.
Figure 13. Increased pro-inflammatory cytokine secretion in HIMEC cells treated with LPS for 1 and 3 hours

HIMEC cells were cultured on a 96-well plate in MCDB 131 medium containing 20% FBS. On day 2 after seeding, wells were replaced with fresh media containing 2.5% FBS. Cells were treated with 0.1 or 1 µg/mL LPS for 1 or 3 h on day 3. Supernatants of the treated cells were analyzed for pro- and anti-inflammatory cytokines using the Human Proinflammatory 7-Plex Ultra-Sensitive Kit measured by the Sector Imager 2400A. Absolute concentrations of (A) IL-1β, (B) IL-10, (C) IFNγ, (D) IL-12p70, (E) TNFα, (F) IL-6 and (G) IL-8 induced by 0.1 or 1 µg/mL LPS are shown. Results are expressed as mean ± Std dev (n=3). *p<.05, **p<.01 vs VC (Vehicle control)
2.2.3.1 Time-Dependent Decrease of LPS-Induced IL-8 Secretion by Compounds B, C and D at 4 and 12 Hours in Caco-2 Cells

To determine if the novel MAO B inhibitors would reduce pro-inflammatory cytokine secretion, IL-6, IL-8, TNFα and IL-1β levels were measured in supernatants of Caco-2 and HIMEC cells stimulated with LPS with or without the novel MAO B inhibitors.

Treatment with 1 µg/mL LPS at 4 and 12 hours increased IL-8 secretion in Caco-2 cells significantly (Figure 14). At 4 hours, baseline IL-8 levels (3.02 pg/mL) increased with LPS (43.4 pg/mL) (Figure 14D). At 12 hours, IL-8 (8.04 pg/mL) increased with LPS (76.2 pg/mL) (Figure 14D). Also, Caco-2 vehicle control cells and treatment with 20 µM of deprenyl, compounds A, B, C or D alone did not significantly increase IL-1β (Figure 14A), IL-6 (Figure 14B), TNFα (Figure 14C) and IL-8 (Figure 14D) indicating that the novel compounds do not upregulate these pro-inflammatory cytokines.

Figure 14. Novel MAO B Inhibitors and deprenyl at 20 µM do not increase pro-inflammatory cytokines while LPS increases IL-8 levels in Caco-2 cells at 4 and 12 hours
Caco-2 cells were cultured on 96-well plates. On Day 3 after seeding, serum concentration was reduced from 10% to 2.5%. On Days 4 and 5, cells were treated with vehicle control (VC), 20 µM of deprenyl, 20 µM of novel MAO B inhibitors or 1 µg/mL LPS alone for 4 or 12 hours. Supernatants were analyzed for (A) IL-1β, (B) IL-6, (C) TNFα or (D) IL-8 secretion. Absolute pro-inflammatory cytokine concentrations induced by these treatments are shown. n=3. *p<.05, **p<.01, ***p<.001 vs VC
In Figure 15 and all applicable, subsequent figures (as indicated by the figure legends), data is presented as absolute secretion of LPS-induced pro-inflammatory cytokines beyond the corresponding controls. Deprenyl and compound A at 20 µM were not effective at reducing IL-8 secretion induced by LPS treatment in Caco-2 cells (Figure 15). At 4 hours, IL-8 was secreted with 1 µg/mL LPS (40.4 pg/mL) and reduced by 20 µM compound B (18.1 pg/mL), compound C (21.0 pg/mL) and compound D (25.0 pg/mL). At 12 hours, IL-8 secretion was induced with 1 µg/mL LPS (68.1 pg/mL) and reduced by 20 µM compound B (40.2 pg/mL), compound C (48.9 pg/mL) and compound D (39.9 pg/mL).

Figure 15. Compounds B, C and D reduce LPS-induced IL-8 secretion in Caco-2 cells at 4 and 12 hours
Caco-2 cells were cultured on 96-well plates. On Day 3 after seeding, serum concentration was reduced from 10% to 2.5%. On Day 4 or 5, cells were treated with 1 µg/mL LPS alone (VC) or 1 µg/mL LPS + 20 µM deprenyl, compounds A, B, C, or D for 4 or 12 hours. Supernatants were analyzed for IL-8 secretion. Absolute IL-8 concentration induced by LPS alone (VC) or reduced by novel MAO B inhibitors is shown (baseline cytokine secretion subtracted from all groups). n=3. *p<.05, **p<.01, ***p<.001 vs VC
2.2.3.2 Concentration-Dependent Decrease of LPS-Induced IL-8 Secretion by Compounds B, C and D in Caco-2 Cells

Since compounds B, C and D were effective at reducing LPS-induced IL-8 secretion in Caco-2 cells at 4 and 12 hours, these three compounds were investigated further to determine concentration dependent reduction of IL-8 by these drugs. Since maximal effects were seen at 12 hours, we selected this time point.

Caco-2 cells were treated with 40 µM deprenyl or the novel compounds for 12 hours which did not increase IL-6, IL-8, TNFα and IL-1β basal levels while 1 µg/mL LPS increased IL-8 secretion (data not shown). Compounds B, C and D reduced LPS-induced IL-8 secretion from Caco-2 cells in a concentration-dependent manner at 12 hours (Figure 16A). LPS-treated Caco-2 cells at 12 hours secreted IL-8 (45.7 pg/mL) and the level of secretion was reduced with compound B at 10 µM (36.4 pg/mL) and 20 µM (29.2 pg/mL). Compound C decreased IL-8 secretion induced by LPS at 5 µM (28.2 pg/mL), 10 µM (26.0 pg/mL) and 20 µM (29.2 pg/mL). Finally, 20 µM of compound D effectively decreased IL-8 secretion (26.9 pg/mL). Interestingly, 0.1 µM of compound D also decreased IL-8 secretion significantly (36.0 pg/mL).

An additional assay was run to assess if higher concentrations of deprenyl and compound A effectively reduced IL-8 secretion induced by LPS in Caco-2 cells. At 12 hours, IL-8 secretion induced by LPS (45.7 pg/mL) was significantly decreased by 40 µM of deprenyl (31.2 pg/mL) and 40 µM compound A (35.0 pg/mL) (Figure 16B).

All novel MAO-B inhibitors clearly decreased IL-8 secretion induced by LPS in Caco-2 cells (Figure 16). Specifically, compounds B, C and D reduced LPS-induced IL-8 secretion in a concentration-dependent manner and 40 µM compound A and the parent compound, deprenyl are able to decrease secreted IL-8.
2.2.3.3  Time-Dependent Decrease of LPS-Induced IL-8, IL-6 and TNFα Secretion by Novel MAO B Inhibitors at 4 and 12 Hours in HIMEC Cells

HIMEC cells were first treated with vehicle control or 10 µM of deprenyl, compounds A, B, C or D to assess if the drugs alone would change basal cytokine levels. Novel compounds alone did not significantly increase IL-1β (Figure 17A), IL-6 (Figure 17B), IL-8 (Figure 17C) and TNFα (Figure 17D) levels. In contrast, 1 µg/mL LPS at 4 and 12 hours increased IL-6, IL-8 and TNFα secretion in HIMEC cells significantly out of the four observed cytokines (Figure 17). At 4 hours, IL-6 levels (6.32 pg/mL) increased with 1 µg/mL of LPS (658 pg/mL). At 12 hours, IL-6 levels (23.5 pg/mL) increased with 1 µg/mL of LPS (2200 pg/mL) (Figure 17B). IL-8 baseline levels (2240 pg/mL) increased with 1 µg/mL LPS at 4 hours (18400 pg/mL) (Figure 17C). At 12 hours, IL-8 (10700 pg/mL)
increased with 1 µg/mL LPS (61500 pg/mL) (Figure 17C). At 4 hours, TNFα (0.14 pg/mL) increased with 1 µg/mL of LPS (5.47 pg/mL) (Figure 17D). At 12 hours, TNFα (1.54 pg/mL) increased with 1 µg/mL of LPS (7.88 pg/mL) (Figure 17D).

Since three of the four cytokines measured were significantly increased with LPS at 4 and 12 hours, reduction of IL-6, IL-8 and TNFα with deprenyl and the novel compounds were further investigated at these two time points.

Figure 17. Novel MAO B inhibitors and deprenyl at 10 µM do not increase pro-inflammatory cytokine secretion while 1 µg/mL of LPS increases IL-6, IL-8 and TNFα levels in HIMEC cells at 4 and 12 hours. HIMEC cells were cultured on 96-well plates. On Day 1 after seeding, serum concentration was reduced from 20% to 2.5%. On Days 2 and 3, cells were treated with vehicle control (VC), 10 µM of deprenyl, 10 µM of novel MAO B inhibitors or 1 µg/mL LPS alone for 4 or 12 hours. Supernatants were analyzed for (A) IL-1β, (B) IL-6, (C) IL-8 or (D) TNFα secretion. Absolute pro-inflammatory cytokine concentrations induced by these treatments are shown. n=3. *p<.05, **p<.01, ***p<.001 vs VC.
Deprenyl and all novel MAO B inhibitors reduced 1 µg/mL LPS-induced IL-8, IL-6 and/or TNFα secretion in HIMEC cells (Figure 18). 1 µg/mL LPS-induced IL-8 secretion (16100 pg/mL) was reduced by 10 µM compound B (8730 pg/mL) and compound C (13600 pg/mL) at 4 hours (Figure 18A). At 12 hours, IL-8 secretion was induced by 1 µg/mL LPS (50800 pg/mL) and decreased with 10 µM compound B (26500 pg/mL) (Figure 18A). At 4 hours, IL-6 secretion was induced by 1 µg/mL LPS (652 pg/mL) and decreased by 10 µM compound A (527 pg/mL), compound B (203 pg/mL), compound C (515 pg/mL) and compound D (443 pg/mL) (Figure 18B). At 12 hours, IL-6 was induced by 1 µg/mL LPS (2170 pg/mL) and reduced by 10 µM compound B (670 pg/mL) and compound D (1690 pg/mL) (Figure 18B). At 4 hours, TNFα secretion was induced by 1 µg/mL LPS (5.33 pg/mL) and decreased by 10 µM deprenyl (3.97 pg/mL), compound A (3.49 pg/mL), compound B (1.45 pg/mL), compound C (3.02 pg/mL) and compound D (2.86 pg/mL) (Figure 18C). At 12 hours, TNFα was induced by 1 µg/mL LPS (6.34 pg/mL) and was decreased by 10 µM compound B (1.98 pg/mL) (Figure 18C).

Since all compounds and deprenyl were effective at regulating LPS-induced pro-inflammatory cytokine expression at 4 hours, deprenyl, compounds A, B, C and D were investigated further at this time point to assess the concentration-dependent reduction of IL-8, IL-6 and TNFα.
Figure 18. Novel MAO-B inhibitors reduce LPS-induced pro-inflammatory cytokine secretion at 4 and 12 hours in HIMEC cells.
HIMECs were cultured on 96-well plates. On Day 1 after seeding, serum concentration was reduced from 20% to 2.5%. On Day 2 or 3, cells were treated with 1 µg/mL LPS alone (VC) or 1 µg/mL LPS + 10 µM deprenyl or compounds A, B, C, or D for 4 or 12 hours. Supernatants were analyzed for (A) IL-8, (B) IL-6 and (C) TNFα secretion. Absolute pro-inflammatory cytokine concentrations induced by 1 µg/mL LPS alone (VC) or reduced by deprenyl or novel MAO B inhibitors is shown (baseline cytokine secretion subtracted from all groups). n=3. *p<.05, **p<.01, ***p<.001 vs VC.
Concentration-Dependent Decrease of LPS-Induced IL-8, IL-6 and TNFα Secretion by Novel MAO B Inhibitors in HIMEC Cells

HIMEC cells treated with 40 µM deprenyl or the novel MAO B inhibitors for 4 hours were not associated with changes in IL-6, IL-8, TNFα and IL-1β expression while 1 µg/mL LPS increased IL-6, IL-8 and TNFα secretion (data not shown). Only compounds B and C at the highest concentration reduced LPS-induced IL-8 secretion from HIMEC cells at 4 hours (Figure 19A). 1 µg/mL LPS-treated HIMEC cells at 4 hours secreted IL-8 (12700 pg/mL) and LPS-induced IL-8 secretion was decreased with 40 µM compound B (7530 pg/mL) and compound C (7960 pg/mL) (Figure 19A).

HIMEC cells secreted IL-6 (574 pg/mL) at 4 hours with 1 µg/mL LPS and secretion was reduced by compound B at 10 µM (488 pg/mL), 20 µM (463 pg/mL) and 40 µM (240 pg/mL) (Figure 19B). Compound C decreased IL-6 secretion induced by LPS at 20 µM (460 pg/mL) and 40 µM (232 pg/mL). Compound D decreased LPS-induced IL-6 secretion at 10 µM (429 pg/mL), 20 µM (366 pg/mL) and 40 µM (256 pg/mL) (Figure 19B).

All compounds and deprenyl at 1, 10, 20 and 40 µM decreased TNFα secretion induced by 1 µg/mL LPS (Figure 19C). At 4 hours, HIMEC cells treated with 1 µg/mL LPS secreted TNFα (17.7 pg/mL) and levels were reduced by deprenyl at 1 µM (4.40 pg/mL), 10 µM (5.91 pg/mL), 20 µM (6.81 pg/mL) and 40 µM (5.45 pg/mL) (Figure 19C). LPS-induced TNFα secretion was reduced by compound A at 1 µM (5.14 pg/mL), 10 µM (7.71 pg/mL), 20 µM (4.79 pg/mL) and 40 µM (6.48 pg/mL) (Figure 19C). LPS-induced TNFα secretion was reduced by compound B at 1 µM (5.95 pg/mL), 10 µM (2.73 pg/mL), 20 µM (3.55 pg/mL) and 40 µM (0.63 pg/mL) (Figure 19C). LPS-induced TNFα secretion was reduced by compound C at 1 µM (4.33 pg/mL), 10 µM (6.54 pg/mL), 20 µM (1.87 pg/mL) and 40 µM (2.17 pg/mL) (Figure 19C). LPS-induced TNFα secretion was reduced by compound D at 1 µM (5.10 pg/mL), 10 µM (2.83 pg/mL), 20 µM (4.75 pg/mL) and 40 µM (2.08 pg/mL) (Figure 19C).

Data in Figure 19 clearly show that all novel MAO-B inhibitors decrease IL-8, IL-6 and TNFα secretion induced by 1 µg/mL LPS in HIMEC cells at 4 hours. Specifically, compounds B, C and D are more effective at decreasing LPS-induced IL-8 and IL-6 secretion
in a concentration-dependent manner than deprenyl and compound A. Notably, all novel inhibitors and deprenyl are able to reduce TNFα secretion induced by LPS.

Figure 19. Novel MAO-B inhibitors reduce LPS-induced pro-inflammatory cytokine secretion in HIMEC cells in a concentration-dependent manner
HIMECs were cultured on 96-well plates. On Day 2 after seeding, serum concentration was reduced from 20% to 2.5%. On Day 3, cells were treated with 1 µg/mL LPS alone (VC) or 1 µg/mL LPS + 1, 10, 20 or 40 µM deprenyl or compounds A, B, C, or D for 4 hours. Supernatants were analyzed for (A) IL-8, (B) IL-6 and (C) TNFα secretion. Absolute pro-inflammatory cytokine concentrations induced by 1 µg/mL LPS alone (VC) or reduced by deprenyl or novel MAO B inhibitors is shown (baseline cytokine secretion subtracted from all groups). n=3. *p<.05, **p<.01, ***p<.001 vs VC
2.2.3.5 Summary of Novel MAO B Inhibitor Regulation of LPS-Induced Pro-inflammatory Cytokine Secretion

Percent reduction of LPS-induced cytokine secretion by deprenyl and the novel MAO B inhibitors are shown in these two tables and values have been calculated by the following equation:

\[
\text{% reduction} = \left(1 - \frac{\text{cytokine (induced by LPS) reduced by drug (pg/mL)}}{\text{cytokine induced by LPS (pg/mL)}}\right) \times 100
\]

20 µM of compounds B, C and D were effective at decreasing IL-8 secretion in Caco-2 cells induced by 1 µg/mL of LPS at 4 and 12 hours (Table 2A). In HIMEC cells, 10 µM of compound B was the most effective at reducing IL-8, IL-6 and TNFα secretion induced at 4 and 12 hours with 1 µg/mL of LPS (Table 2B).

Table 2. Time-Dependent Reduction of LPS-Induced Cytokine Secretion in Caco-2 Cells after Treatment with Novel MAO B Inhibitors

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Drug conc. 20 µM

Colour Scale (% Reduction relative to LPS-treated [value of 100%])

| 0 | 10 | 20 | 30 | 40 | 50 | 60 | 70 | 80 | 90 | 100 |

NS, non significant. Statistically significant values are shown as percent reduction compared to cells treated with LPS alone. Darker shades of red represent a larger decrease of pro-inflammatory cytokines with deprenyl or novel MAO B inhibitor treatment in (A) Caco-2 cells and (B) HIMEC cells.
Compounds B, C and D decreased IL-8 secretion induced at 12 hours by LPS in a concentration-dependent manner in Caco-2 cells (Table 3A). Compounds B, C and D decreased IL-8 and IL-6 secretion induced by LPS at 4 hours in a concentration-dependent manner in HIMECs (Table 3B). At 4 hours, deprenyl and all novel compounds decreased TNFα secretion induced by 1 µg/mL LPS in HIMEC cells (Table 3B).

Table 3. Concentration-Dependent Reduction of LPS-Induced Cytokine Secretion in Caco-2 Cells after Treatment with Novel MAO B Inhibitors

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12h Treatment

4h Treatment

Table 3. Concentration-Dependent Reduction of LPS-Induced Cytokine Secretion in HIMEC Cells after Treatment with Novel MAO B Inhibitors

<table>
<thead>
<tr>
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4h Treatment

Colour Scale (% Reduction relative to LPS-treated)

| 0 | 10 | 20 | 30 | 40 | 50 | 60 | 70 | 80 | 90 | 100 |

NS, non significant; NA, not available. Statistically significant values are shown as percent reduction compared to cells treated with LPS alone. Darker shades of red represent a larger decrease of pro-inflammatory cytokines with deprenyl or novel MAO B inhibitor treatment in (A) Caco-2 cells and (B) HIMEC cells.
2.2.4 Regulation of LPS-Induced Pro-Inflammatory Cytokine Gene Expression by Novel MAO B Inhibitors in Caco-2 and HIMEC Cells

Based on the cytokine results, we focused on TNFα, IL-6 and IL-8 gene expression analysis using qRT-PCR. We examined in Caco-2 and HIMEC cells the relative change in gene expression induced by LPS and whether the novel MAO inhibitors reduced relative gene expression of the pro-inflammatory cytokines. Relative fold differences were determined and compared to expression of the genes of interest (TNFα, IL-6 and IL-8) in either vehicle control cells or LPS-treated cells as indicated in the figure legends.

2.2.4.1 Time-Dependent Decrease of LPS-Induced IL-8 Gene Expression by Novel MAO B Inhibitors in Caco-2 Cells

Similar to IL-8 secretion, 20 µM of the novel MAO B inhibitors or deprenyl did not affect IL-8 gene expression at 4 hours (Figure 20A) and 12 hours (Figure 20B). LPS induced an increase in IL-8 gene expression over vehicle control cells at 4 hours (9.57 fold, Figure 20A) and 12 hours (2.38 fold, Figure 20B).

![Figure 20. IL-8 gene expression at 4 and 12 hours is not affected by the novel MAO B inhibitors or deprenyl alone but is increased with 1 µg/mL of LPS in Caco-2 cells.](image-url)

Caco-2 cells were cultured on 12-well plates. On Day 2 after seeding, serum concentration was reduced from 10% to 2.5%. On Days 3 and 4, cells were treated with vehicle control, 20 µM of deprenyl, 20 µM of compounds A, B, C or D or 1 µg/mL LPS alone for (A) 4 or (B) 12 hours and total RNA was harvested. Using reverse transcriptase, cDNA was synthesized from 2 µg total RNA and used as a template for qRT-PCR reactions using primers and probes for IL-8 and GAPDH. Each reaction contained cDNA derived from 10 ng total RNA. Results are expressed as mean fold-change relative to vehicle control (value of 1.0 fold change) ± SEM. n=3. *p<.05, **p<.01, ***p<.001
Since IL-8 gene expression in Caco-2 cells was significantly increased by LPS at 4 and 12 hours, IL-8 mRNA levels were evaluated with co-treatment of 1 µg/mL LPS and 20 µM deprenyl or the novel MAO B inhibitors at these time points. At 4 hours, 20 µM compound C reduced gene expression of LPS-induced IL-8 (0.76-fold) compared to the expression in LPS-treated Caco-2 cells (Figure 21A). At 12 hours, LPS-induced IL-8 gene expression was reduced by 20 µM deprenyl (0.78-fold), compound B (0.67-fold), compound C (0.79-fold) and compound D (0.65-fold) compared to the expression in LPS-treated Caco-2 cells (Figure 21B).

![Figure 21](image)

**Figure 21.** Novel MAO-B inhibitors reduce LPS-induced IL-8 gene expression at 4 and 12 hours in Caco-2 cells.

Caco-2 cells were cultured on a 12-well plate. On Day 2 after seeding, serum concentration was reduced from 10% to 2.5%. On Days 3 and 4, cells were treated with 1 µg/mL LPS alone or 1 µg/mL LPS + 20 µM deprenyl, compounds A, B, C or D for (A) 4 or (B) 12 hours and total RNA was harvested. Using reverse transcriptase, cDNA was synthesized from 2 µg total RNA and used as template for qRT-PCR reactions using primers and probes for IL-8 and GAPDH. Each reaction contained cDNA derived from 10 ng total RNA. Results are expressed as mean fold-change relative to cells treated with LPS alone (value of 1.0 fold change) ± SEM. n=3. *p<.05, **p<.01, ***p<.001

### 2.2.4.2 Time-Dependent Decrease of LPS-Induced IL-8, IL-6 and TNFα Gene Expression by Novel MAO B Inhibitors in HIMEC Cells

At 4 and 12 hours, 20 µM of the novel MAO B inhibitors or deprenyl alone did not affect IL-8, IL-6 and TNFα gene expression in HIMEC cells (Figure 22). Treatment with 1 µg/mL LPS increased gene expression of IL-8 at 4 hours (3.54-fold, Figure 22A) and 12 hours (8.60-fold, Figure 22B), IL-6 at 4 hours (89.5-fold, Figure 22C) and 12 hours (64.7-fold, Figure 22D) and TNFα at 4 hours (11.3-fold, Figure 22E) and 12 hours (8.38-fold, Figure 22F) compared to expression in vehicle controls.
Figure 22. IL-8, IL-6 and TNFα gene expression at 4 and 12 hours are not affected by the novel MAO B inhibitors or deprenyl alone but are increased with 1 µg/mL of LPS in HIMEC cells

HIMEC cells were cultured on 12-well plates. On Day 2 after seeding, serum concentration was reduced from 20% to 2.5%. On Days 3 and 4, cells were treated with vehicle control, 20 µM of deprenyl, 20 µM of compounds A, B, C or D or 1 µg/mL LPS alone for (A, C and E) 4 or (B, D and F) 12 hours and total RNA was harvested. Using reverse transcriptase, cDNA was synthesized from 2 µg total RNA and used as a template for qRT-PCR reactions using primers and probes for (A and B) IL-8, (C and D) IL-6 or (E and F) TNFα and GAPDH. Each reaction contained cDNA derived from 10 ng total RNA. Results are expressed as mean fold-change relative to vehicle control (value of 1.0 fold change) ± SEM. n=3. *p<.05, **p<.01, ***p<.001
Since IL-8, IL-6 and TNFα gene expression in HIMEC cells was significantly increased with 1µg/mL LPS at 4 and 12 hours, mRNA levels of each pro-inflammatory cytokine were evaluated with treatment of LPS and deprenyl or the novel MAO B inhibitors at these time points.

At 12 hours, 1 µg/mL LPS-induced IL-8 expression was reduced by 20 µM compound B (0.68-fold) and compound C (0.84-fold) compared to LPS-treated HIMEC cells (Figure 23B). LPS-induced IL-6 gene expression was reduced by 20 µM compound B (0.63-fold) and by compound D (0.65-fold) compared to the expression in LPS-treated HIMEC cells at 4 hours (Figure 23C). At 12 hours, LPS-induced IL-6 gene expression was reduced by 20 µM compound B (0.36-fold), compound C (0.55-fold) and compound D (0.55-fold) compared to the expression in LPS-treated HIMEC cells (Figure 23D). At 4 hours, LPS-induced TNFα gene expression was reduced by 20 µM of compound B (0.59-fold) and compound C (0.60 fold) compared to the expression in LPS-treated cells (Figure 23E). TNFα gene expression induced by LPS was reduced by compound B (0.55-fold) and compound C (0.63-fold) compared to the expression in LPS-treated cells at 12 hours.
Figure 23. Novel MAO-B inhibitors reduce LPS-induced pro-inflammatory cytokine gene expression at 4 and 12 hours in HIMEC cells.

HIMEC cells were cultured on a 12-well plate. On Day 2 after seeding, serum concentration was reduced from 20% to 2.5%. On Days 3 and 4, cells were treated with 1 µg/mL LPS alone or 1 µg/mL LPS + 20 µM deprenyl, compounds A, B, C or D for (A, C and E) 4 or (B, D and F) 12 hours and total RNA was harvested. Using reverse transcriptase, cDNA was synthesized from 2 µg total RNA and used as a template for qRT-PCR reactions using primers and probes for (A and B) IL-8, (C and D) IL-6 or (E and F) TNFα and GAPDH. Each reaction contained cDNA derived from 10 ng total RNA. Results are expressed as mean fold-change relative to cells treated with LPS alone (value of 1.0 fold change) ± SEM. n=3. *p<.05, **p<.01, ***p<.001
2.2.4.3 Summary of Novel MAO B Inhibitor Regulation of LPS-Induced Pro-inflammatory Cytokine Gene Expression

The data presented in Figure 21 and Figure 23 are summarized in Table 4. Fold reduction of LPS-induced pro-inflammatory cytokine gene expression by deprenyl and the novel MAO B inhibitors are shown in this table and values have been calculated by the following equation:

\[
Fold\ reduction = \frac{1}{\text{ratio of (drug + LPS treated cells) to (LPS treated cells)}}
\]

Higher numerical values represent greater fold reduction and more effectiveness of the novel MAO B inhibitor. 20 µM of all compounds except for compound A are effective at reducing IL-8 gene expression in Caco-2 cells induced at 12 hours by 1 µg/mL of LPS (Table 4A). In HIMEC cells, 20 µM of compound B, C and D are the most effective at decreasing IL-8, IL-6 and TNFα gene expression induced at 4 and 12 hours by LPS (Table 4B).

Table 4. Fold Reduction of LPS-Induced Cytokine Gene Expression in Caco-2 and HIMEC Cells after Treatment with Novel MAO B Inhibitors

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<tr>
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<th>Caco-2 Cells</th>
<th>HIMEC Cells</th>
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<tbody>
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<td>IL-6</td>
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<tr>
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<td>12h</td>
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<tr>
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Colour Scale (Fold Reduction relative to LPS-treated [value of 1.0])
1.00 1.25 1.50 1.75 2.00 2.25 2.50 2.75 3.00

NS, non significant. Statistically significant values are shown as fold reduction compared to cells treated with LPS alone. Darker shades of red represent a greater decrease of pro-inflammatory cytokine gene expression with deprenyl or novel MAO B inhibitor treatment in (A) Caco-2 cells and (B) HIMEC cells.
Chapter 3: Discussion

3.1 Relative MAO A and B Protein and Gene Expression in Endothelial and Epithelial Cells

Using Western Blot analysis, Caco-2 and HIMEC cells expressed MAO B and not MAO A protein. However, MAO A mRNA was found in higher levels in Caco-2 cells than MAO B and HIMEC cells expressed MAO A mRNA but not MAO B as concluded by qRT-PCR analysis. Therefore, MAO A and B protein and gene expression do not correlate in either Caco-2 or HIMEC cells. A common assumption of studying mRNA is that it is an informative predictor of protein expression. Multiple correlation analyses of mRNA-protein expression reveal that this relationship (increased mRNA means increased protein) does not exist in the majority of genes analyzed (Gry et al., 2009; Guo et al., 2008). For instance, Gry et al. (2009) and Guo et al. (2008) found that only a third of examined RNA species in 23 human cell lines and half of 71 genes analyzed in isolated monocytes of unrelated women showed significant positive correlation with their corresponding protein levels.

The discrepancy of MAO A mRNA-protein expression in Caco-2 and HIMEC cells may be explained by a number of possible reasons. The cell lines could express limited amounts of MAO A protein that cannot be detected by Western Blot or the protein may have a faster degradation rate than MAO B. Low or nonexistent MAO A protein generation with detectable mRNA levels could mean that there is minimal translation of MAO A transcripts (Imaizumi et al., 2000). The discrepancy of MAO B mRNA-protein correlation could be due to the possibility that MAO B protein is more stable than its RNA counterpart. In addition, MAO B has a long turnover rate of approximately 30 days in the primate brain that may promote a negative feedback mechanism for decreased gene transcription and subsequently, non-detectable levels of MAO B mRNA (Arnett et al., 1987; Guo et al., 2008). Lastly, since HIMEC cells were isolated directly from healthy sites of diverticulitis patients, the primary cell line utilized may have a particular single-nucleotide substitution that may reduce MAO B probe and primer binding, leading to the apparent absence of MAO B mRNA detection.

Previous studies have also demonstrated the specific incongruity of MAO A and MAO B mRNA-protein expression in both in vitro and in vivo studies (Shih et al., 1999; Sivasubramaniam et al., 2003). Shih et al. (1999) demonstrated that confluent Caco-2 cells
exhibit high levels of MAO B and not MAO A protein. Sivasubramaniam et al. (2003) revealed that mRNA levels of both MAO isotypes did not correspond with protein expression at various tissue sites of the body. In vitro immunostaining experiments of Caco-2 cells performed in our research group also confirmed that MAO B is expressed and not MAO A (Goebeler, V, personal communication).

Only a handful of efforts have found positive correlation between mRNA-protein expression levels and since proteins are the direct facilitators of cellular processes, studying protein expression is more likely to reflect function than mRNA (Greenbaum et al., 2003). In the present study, low correlation of mRNA-protein expression indicates that MAO A and B are controlled at both translational and transcriptional levels. Therefore, mRNA and protein expression must be studied separately to achieve a better understanding of the role of MAO function in future investigations.

3.2 Novel MAO B Inhibitor Effect on Intestinal Epithelial and Endothelial Cell Viability, Cytotoxicity and Apoptosis

The novel MAO B inhibitors tested in the current study do not decrease Caco-2 and HIMEC cell viability, nor increase cell cytotoxicity or apoptosis and are within the range of MAO inhibitor concentrations used in other in vitro studies (Abakumova et al., 1998; Maher and Davis, 1996; Seymour et al., 2003; Youdim and Bakhle, 2006). Although no statistically significant differences of Caco-2 cell viability were detected with treatment of the novel compounds, there was variability in the data (Figure 11). We addressed this concern by repeating the cell viability analysis in HEK 293 cells. The Cell-Titre Blue Viability Assay confirmed that multiple concentrations of compound A do not decrease cell viability in HEK 293 cells (Appendix). In addition, our research group has confirmed that doses of up to 20 mg/kg of compounds B and D did not induce toxicity in C57BL/6 mice when administered intravenously, subcutaneously or orally (Putnins, EE, personal communication). These results demonstrate the clinical potential and applicability of the novel MAO B inhibitors in other experimental models in the future.

It has been revealed extensively in the past that deprenyl possesses anti-apoptotic properties (Carrillo et al., 2000; Magyar, 2011; Mandel et al., 2005; Qin et al., 2003; Saura et
Mandel et al., (2005) show that the anti-apoptotic effects of deprenyl contribute to its neuroprotective effects. Deprenyl also decreases apoptosis in rat ischemia reperfusion and cardiac tissue (Qin et al., 2003; Toronyi et al., 2002). Magyar (2011) determined deprenyl exhibits anti-apoptotic characteristics at specific concentrations: lower nanomolar concentrations of deprenyl promoted anti-apoptosis while concentrations higher than 10 µM induced apoptosis. Contrary to deprenyl, compound D exhibited anti-apoptotic effects at concentrations of 20 and 40 µM, which were confirmed with multiple, cell-based and enzymatic assays in the Putnins laboratory (data not shown). Future studies focusing on the anti-apoptotic properties of the novel MAO B inhibitors would be a unique path to pursue as inhibition of caspases are implicated in Huntington’s disease (Graham et al., 2011; Ooi et al., 2014).

3.3 Epithelial and Endothelial Cell Cytokine Response to Novel MAO B Inhibitor Treatment

In the present study, the four novel MAO B inhibitors decreased LPS-induced pro-inflammatory cytokine gene expression and secretion in Caco-2 and HIMEC cells. Besides their original use to treat neurological diseases, clinically available MAO inhibitors have been recently shown to downregulate pro-inflammatory cytokines in various systems (Bielecka et al., 2010; Ekuni et al., 2009; Lin et al., 2000; Martino et al., 2012; Muller et al., 1998; Salter-Cid et al., 2005; Senini, 2011). Salter-Cid et al. (2005) and Ekuni et al. (2009) demonstrated that inhibition of amine oxidases decreased IL-6 and TNFα levels in LPS-treated mice. Bielecka et al. (2010) showed that moclobemide diminished LPS-stimulated IL-1β mRNA in a mixed glial cell culture. Similarly, Senini (2011) demonstrated that deprenyl decreased LPS-induced TNFα expression in Madin-Darby canine kidney epithelial cells. Moclobemide also reduced TNFα and IL-8 levels in LPS-stimulated whole blood and selegiline reduced TNFα in peripheral blood mononuclear cells (Lin et al., 2000; Muller et al., 1998).

MAO inhibitors are currently used to target MAO as a molecular biomarker of various pathologies and are now known as polypharmacologic drugs as the focus of its activity are directed to tissue sites other than the brain (Carradori and Petzer, 2015; Wu et al.,
Carradori and Petzer (2015) reviewed multiple patents that demonstrate the use of MAO inhibitors including phenelzine, selegiline and moclobemide as treatment for hair loss, prostate cancer, muscle dystrophy, skin neoplasms and inflammation. However, deprenyl was shown to increase IL-2 and IFNγ in mice and at high concentrations, phenelzine aggravates the inflammatory response in microglia cells (Chung et al., 2012; Thyagarajan et al., 2013).

With the data presented in this study, it is clear that the four novel MAO B inhibitors fit a drug profile that has not been explored in depth but is of particular interest in the current literature. Since the novel inhibitors reduce multiple pro-inflammatory cytokine expression in LPS-induced intestinal cell lines and do not cross the BBB, they present a viable option for treatment of inflammatory diseases compared to other MAO inhibitors explored so far. Since compounds A, B, C and D reduce IL-8, IL-6 and TNFα, these inhibitors have the potential to diminish pro-inflammatory cytokine function of perpetuating the inflammatory process by decreasing neutrophil recruitment, inhibiting B-cell stimulation and reducing apoptosis. Furthermore, it is crucial to explore potential mechanisms by which the novel MAO B inhibitors can reduce harmful immune response mediators in certain mucosal inflammatory diseases. In fact, our research group is currently using a DSS-induced colitis mouse model to determine the effect of the novel compounds on colonic tissue and plasma cytokine expression.

3.3.1 Potential Mechanism of Novel MAO B Inhibitor Reduction of Pro-Inflammatory Cytokine Expression

Since the novel MAO B inhibitors downregulate LPS-induced pro-inflammatory cytokine protein and gene expression, the drugs can potentially reduce cytokine expression at either the transcriptional or protein secretion level.

3.3.1.1 Novel MAO B Inhibitor Effect on Pro-Inflammatory Cytokine Gene Expression

The NF-κB pathway is considered the prototypical signaling pathway of inflammation because cytokine, chemokine and adhesion molecule gene expression is reliant
on the role of the transcription factor NF-κB (Lawrence, 2009). NF-κB consists of the inactive p50/p65 heterodimer, which is kept in the cytoplasm by IκB (Blaecke et al., 2002; Jobin and Sartor, 2000). Mediators like LPS increase ROS from cellular mitochondria (through MAO activity) and H₂O₂ generation, which induce IKK and subsequent NF-κB activation. Therefore, inhibition of mitochondrial MAO B by the novel compounds could decrease LPS-induced ROS and ROS-associated molecules and reduce NF-κB activation and gene expression of pro-inflammatory cytokines (Figure 24B). The novel MAO B inhibitors may inhibit active NF-κB subunit translocation into the nucleus. This may be possible by inhibiting IKK activity or prohibiting IκB itself to unbind the NF-κB active subunits thereby preventing its entry into the nucleus (Figure 24B). MAO inhibitors could also inhibit the active unit in the cytoplasm or nucleus to prevent activation of NF-κB (Figure 24B). However, because not all novel MAO B inhibitors demonstrated effectiveness of decreasing IL-8, IL-6 and TNFα mRNA levels, the novel drugs could reduce cytokine secretion as well.

Figure 24. Proposed mechanism of novel MAO B inhibitor action
MAO B is localized in the outer mitochondrial membrane and can be targeted by our novel MAO B inhibitors (arrow). Proposed mechanisms of novel MAO B inhibitor action (question marks) to decrease pro-inflammatory cytokine secretion (A) or gene expression (B) induced by LPS are shown. Adapted from Sun et al., 2013 and Probert et al., 2000
3.3.1.2 Effect of Novel MAO B Inhibitors on Pro-Inflammatory Cytokine Secretion

The novel MAO B inhibitors could abrogate the constitutive exocytosis pathway of IL-8, IL-6 and TNFα by inhibiting vesicular transfer of the cytokines to the cell surface (Figure 24A). In addition, ROS activates TACE, releasing soluble TNFα from its membrane-bound form. Inhibiting MAO B activity and subsequent decrease of ROS levels by novel MAO B inhibitors could reduce TACE activation and therefore TNFα secretion (Figure 24A). Another possibility could be direct inhibition of TACE by the novel drugs, prohibiting the conversion of TNFα to its soluble form.

Novel MAO B inhibitor alteration of NF-κB activity and pro-inflammatory cytokine secretion are possible mechanisms of the compounds. However, the novel MAO B inhibitors, especially compounds B and D, must be further investigated individually to determine specific mechanistic actions of these drugs.

3.4 MAO B Inhibitors on Chronic Inflammation

Similar to the novel MAO B inhibitors presented in this study, alterations of chemical groups of deprenyl to create potent and selective MAO B inhibitors have been done before (Magyar, 2011). However, these novel drugs that do not cross the BBB target the inflammatory process, a property of MAO inhibitors that has not been explored in depth previously. Compounds B and D are readily absorbed and available through subcutaneous, intravenous and oral administrations in mouse models showing promise of their clinical use to treat inflammatory diseases (Putnins, EE, personal communication).

Drugs targeting amine oxidases have shown effectiveness at dampening inflammation in vivo. MAO inhibition reduces cardiovascular disease and moclobemide attenuates cyclic GMP, endothelial nitric oxide release and therefore endothelial cell dysfunction in the mouse aorta (Machado-Vieira and Mallinger, 2012; Sturza et al., 2013). Semicarbazide-sensitive amine oxidases (SSAOS), also known as vascular adhesion proteins, are enzymes that specifically metabolize primary amines like benzylamines, a target shared with MAO B (Kinemuchi et al., 2004; Lin et al., 2011). Similar to MAOs, SSAO activity produces toxic aldehyde and H₂O₂ and mediates the interaction between leukocytes and activated endothelial cells (Salter-Cid et al., 2005). Salter-Cid et al. (2005) found that SSAO inhibitors
reduced mortality, ulceration scores and colonic cytokine and serum levels of IL-6 and TNFα in a UC mouse model induced by LPS and prolonged survival in LPS-induced endotoxemia. The effectiveness of inhibiting amine oxidases and mediators of inflammation are shared characteristics of the novel MAO B inhibitors that may improve inflammatory states, specifically in the GI tract.

The connection and involvement of IBD with other inflammatory states of the body, such as asthma and psoriasis, has been readily established in the past (Alpsoy et al., 1998; D'Andrea et al., 2010; Pietrzak et al., 2009). Pietrzak et al. (2009) found gastroduodenitis treatment added to topical antipsoriatic therapy improved symptoms of a patient with exudative psoriasis. Alpsoy et al. (1998) showed that moclobemide could be used as an adjunct with corticosteroid ointment to treat psoriasis. Therefore, a possible application of the novel MAO B inhibitors could target not only IBDs but also other inflammatory diseases such as chronic conditions of the skin. Novel inhibitors to target inflammation within the GI tract are currently being synthesized and approved. For example, monoclonal antibodies against TNFα (adalimumab or Humira), and a4b7 integrin (vedolizumab or Entyvio) are used to target IBDs by reducing neutrophil infiltration (Raine, 2014; Rutgeerts et al., 2004). Amaryllidaceae alkaloids reverse calprotectin (proteins abundant in inflammatory states) properties of growth-inhibition and apoptosis that could also be used to improve IBD (Waugh et al., 2013; Yui et al., 2003). Reduction in severity of other chronic inflammatory diseases has also been established with monoamine oxidase inhibitor treatments such as rasagiline in microglial cells and phenelzine in IBD and periodontitis (Carradori and Petzer, 2015; Ekuni et al., 2009; Kast, 1998; Trudler et al., 2014). Therefore, since the novel MAO B inhibitors are able to reduce LPS-induced pro-inflammatory cytokine expression and do not cross the BBB to induce CNS effects like their preexisting counterparts, they fit into a profile of new drugs that can potentially target mucosal inflammatory diseases.
3.5 Conclusions

This study provides evidence that four novel MAO B inhibitors, derived from deprenyl, reduce pro-inflammatory cytokine expression in gut-associated epithelial and endothelial cell lines. Specifically:

1) The four novel MAO B inhibitors neither decreased cell viability nor increased cytotoxicity and apoptosis in Caco-2 and HIMEC cells, although compound D above 20 µM decreased apoptosis in Caco-2 cells.

2) The four novel MAO-B inhibitors were more effective than deprenyl at reducing LPS-induced pro-inflammatory cytokine secretion in Caco-2 and HIMEC cells. In Caco-2 cells, compounds B, C and D decreased LPS-induced IL-8. In HIMEC cells, compounds B and C decreased LPS-induced IL-8 while all compounds decreased LPS-induced IL-6 and TNFα secretion.

3) The four novel MAO B inhibitors reduced LPS-induced pro-inflammatory cytokine gene expression in Caco-2 and HIMEC cells. In Caco-2 cells, compounds A, B, C, D and deprenyl reduced LPS-induced IL-8 gene expression. In HIMEC cells, compound D decreased LPS-induced IL-6 gene expression while compounds B and C reduced LPS-induced IL-8, TNFα and IL-6 gene expression.

In conclusion, the anti-inflammatory properties of novel MAO-B inhibitors that have reduced penetration across the BBB make them therapeutic candidates for GI mucosal diseases like IBDs. Reduction of pro-inflammatory cytokines that are important in many chronic inflammatory disorders expands the potential use of these novel MAO B inhibitors for other diseases. Elucidating the mechanism by which these novel MAO B inhibitors act and their effect on pro-inflammatory cytokines in in vivo models needs to be further investigated.
3.6 Future Directions

1) Investigate the effect of the novel MAO B inhibitors on pro-inflammatory cytokine levels in a DSS-induced or Muc-2 deficient colitis mouse model. Serum and colonic tissue samples could be collected from diseased mice treated with or without the novel compounds and analyzed for pro-inflammatory cytokines or immune cell infiltrates.

2) Investigate an *in vitro* mechanism by which novel MAO B inhibitors reduce pro-inflammatory cytokine expression. Inhibitors of transcription, protein synthesis and cytokine secretion (i.e. TACE inhibitors) could be used to determine the mechanism of novel MAO B inhibitor-mediated reduction of LPS-induced pro-inflammatory cytokines.

3) The anti-apoptotic properties of the novel MAO B inhibitors have been confirmed with *in vitro* caspase activity assays and should be further evaluated.
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Appendix

Figure 25. Compound A demonstrates limited variability with the Cell-Titre Blue Viability assay
J.Terc of CDRD tested compound A and deprenyl on HEK 293 cells and it confirmed that the variability of cell viability is not statistically significant compared to vehicle control (VC) cells.