DOWNREGULATION OF LIPOPOLYSACCHARIDE-INDUCED INTESTINAL EPITHELIAL CELL CHEMOKINE SECRETION BY A NOVEL MAO B INHIBITOR

by

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Downregulation of Lipopolysaccharide-Induced Intestinal Epithelial Cell Chemokine Secretion by a Novel MAO B Inhibitor

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Abstract

Lipopolysaccharide (LPS) is a membrane component of Gram-negative bacteria. Enzyme monoamine oxidase B (MAO B) gene and protein expression was upregulated in diseased tissue of LPS-induced periodontal mucosal chronic inflammation in rat; MAO inhibition with phenelzine reduced signs of inflammation (Ekuni et al., 2009). Current MAO inhibitors are brain permeable and clinically used to treat CNS-associated diseases. Interestingly, some of them showed promising peripheral effects. To manage chronic non-CNS inflammation, Dr. Putnins and collaborators redeveloped novel MAO B inhibitors with reduced blood-brain barrier permeability. These novel inhibitors have significantly reduced LPS-induced cytokine gene and protein expression in various epithelial and endothelial cell models via unknown mechanisms. We investigated the possible signaling pathways mediating LPS-induced interleukin-8 (IL-8) protein expression, and the mechanism by which novel MAO B inhibitor Compound B reduced such effects in intestinal epithelial cell line Caco-2 cells. Caco-2 cell differentiation was induced by culturing in Transwell-inserts for extended culture times. MAO B protein expression, levels of IL-8 secretion, total NF-κB p65 nuclear translocation, phosphorylation of NF-κB p65 at Ser536 and phosphorylation of ERK1/2, p38 and JNK MAPKs were assessed following stimulation by LPS with or without Compound B in differentiated and undifferentiated Caco-2 cultures via immunoprecipitation, ELISA, immunofluorescence and Western blotting. We showed that LPS-induced IL-8 secretion in Caco-2 cultures was independent of the common LPS-induced signaling pathways including NF-κB p65 nuclear translocation and phosphorylation of NF-κB p65 and MAPKs. MAO B protein expression was only detected in differentiated Caco-2 cultures. Compound B exhibited equal efficacy at downregulating LPS-induced IL-8 secretion in both undifferentiated and differentiated Caco-2 cultures. In summary,
IL-8 downregulation by Compound B appears to be independent of the presence of MAO B protein, suggesting a possible off-target effect.
Lay Summary

Inhibition of enzyme monoamine oxidase B (MAO B) has demonstrated therapeutic potential in the management of mucosal chronic inflammation. Current MAO B inhibitors are brain-penetrative thus not suitable for treating diseases outside the brain. Dr. Putnins and colleagues redeveloped a novel MAO B inhibitor Compound B with low brain entry and proven anti-inflammatory effect in various mucosal inflammatory disease models. Two intestinal epithelial cell models with and without MAO B were stimulated with bacterial cell wall component lipopolysaccharide (LPS) to induce the release of pro-inflammatory molecule interleukin-8 (IL-8). The mechanisms of IL-8 induction by LPS and its suppression by Compound B were investigated. LPS induced IL-8 release via mechanisms independent of what is classically known. Compound B suppressed LPS-induced IL-8 release in both models equally by 30%, suggesting a suppressing mechanism independent of the presence of MAO B.
Preface

This thesis is original, independent, unpublished, and prepared entirely by the author X. Wang. It describes a research project conducted in the Putnins Laboratory at the Department of Oral Biological and Medical Sciences, Faculty of Dentistry at the University of British Columbia. The project is part of a collaboration between the Putnins Laboratory and the Centre for Drug Research and Development (CDRD). Dr. E. Putnins and Dr. V. Goebeler participated in this project as the principal supervisor and research manager, providing support in terms of defining aims, experimental design and thesis editing. All the experiments were performed, data were analyzed and figures were created entirely by X. Wang except for the following:

Chapter 1. Figure 1.1, 1.2, and 1.5 to 1.11 were used with permission from applicable sources.

Chapter 1. Figure 1.3 and 1.4. The experiments were performed and data were analyzed by C. Kim from CDRD.

Chapter 2. Figure 2.1 was adapted from Corning Inc. and modified without altering the original meaning of the content.
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<th>Abbreviation</th>
<th>Description</th>
</tr>
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<tbody>
<tr>
<td>AP-1</td>
<td>activator protein-1</td>
</tr>
<tr>
<td>AUC&lt;sub&gt;last&lt;/sub&gt;</td>
<td>area under the plasma concentration-time curve from time zero to time of last measurable concentration</td>
</tr>
<tr>
<td>BBB</td>
<td>blood-brain barrier</td>
</tr>
<tr>
<td>C/EBP</td>
<td>CCAAT-enhancer-binding protein</td>
</tr>
<tr>
<td>Caco-2</td>
<td>human colon colorectal adenocarcinoma epithelial cell line</td>
</tr>
<tr>
<td>CD14</td>
<td>cluster of differentiation 14</td>
</tr>
<tr>
<td>CDRD</td>
<td>Center for Drug Research and Development</td>
</tr>
<tr>
<td>C&lt;sub&gt;max&lt;/sub&gt;</td>
<td>maximum (peak) plasma drug concentration</td>
</tr>
<tr>
<td>CNS</td>
<td>central nervous system</td>
</tr>
<tr>
<td>E. coli</td>
<td><em>Escherichia coli</em></td>
</tr>
<tr>
<td>ELISA</td>
<td>enzyme-linked immunosorbent assay</td>
</tr>
<tr>
<td>ERK</td>
<td>extracellular signal-regulated kinase</td>
</tr>
<tr>
<td>FBS</td>
<td>fetal bovine serum</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>granulocyte-macrophage colony-stimulating factor</td>
</tr>
<tr>
<td>HepG2</td>
<td>human liver hepatocellular carcinoma epithelial cell line</td>
</tr>
<tr>
<td>IC&lt;sub&gt;50&lt;/sub&gt;</td>
<td>concentration of drug producing 50% inhibition</td>
</tr>
<tr>
<td>IFN</td>
<td>interferon</td>
</tr>
<tr>
<td>IKK</td>
<td>IκB kinase</td>
</tr>
<tr>
<td>IL</td>
<td>interleukin</td>
</tr>
<tr>
<td>IP</td>
<td>immunoprecipitation</td>
</tr>
<tr>
<td>IRAK</td>
<td>interleukin-1 receptor associated kinases</td>
</tr>
<tr>
<td>IRF</td>
<td>interferon regulatory factor</td>
</tr>
<tr>
<td>IκB</td>
<td>inhibitor of kappa B</td>
</tr>
<tr>
<td>JNK</td>
<td>c-Jun N-terminal kinase</td>
</tr>
<tr>
<td>K&lt;sub&gt;p&lt;/sub&gt;</td>
<td>equilibrium distribution ratio of substance between a tissue and blood</td>
</tr>
<tr>
<td>LBP</td>
<td>LPS-binding protein</td>
</tr>
<tr>
<td>LC-MS</td>
<td>liquid chromatography-mass spectrometry</td>
</tr>
<tr>
<td>LPS</td>
<td>lipopolysaccharide</td>
</tr>
<tr>
<td>Mal/TIRAP</td>
<td>MyD88 adaptor-like protein/TIR domain-containing adaptor protein</td>
</tr>
<tr>
<td>MAO</td>
<td>monoamine oxidase</td>
</tr>
<tr>
<td>MAOI</td>
<td>monoamine oxidase inhibitor</td>
</tr>
<tr>
<td>MAPK</td>
<td>mitogen-activated protein kinase</td>
</tr>
<tr>
<td>MAPKK/MEK</td>
<td>mitogen-activated protein kinase kinase</td>
</tr>
<tr>
<td>MCP</td>
<td>monocyte chemoattractant protein</td>
</tr>
<tr>
<td>MD-2</td>
<td>myeloid differentiation factor-2</td>
</tr>
<tr>
<td>MEKK</td>
<td>mitogen-activated protein kinase kinases kinase</td>
</tr>
<tr>
<td>MyD88</td>
<td>myeloid differentiation primary response protein 88</td>
</tr>
<tr>
<td>NF-IL-6</td>
<td>nuclear factor for IL-6 expression</td>
</tr>
<tr>
<td>NF-κB</td>
<td>nuclear factor kappa-light-chain-enhancer of activated B cells</td>
</tr>
<tr>
<td>PAMP</td>
<td>pathogen-associated molecular pattern</td>
</tr>
<tr>
<td>Raw264.7</td>
<td>mouse Abelson murine leukemia virus-transformed macrophage</td>
</tr>
</tbody>
</table>
cell line
ROS reactive oxygen species
SILAC stable isotope labeling with amino acids in cell culture
TAK TGF-β-activated kinase
TIR Toll/IL-1R
TLR Toll-like receptor
TNF tumor necrosis factor
TRAF tumor necrosis factor receptor associated factor
TRAM TRIF-related adaptor molecule
TRIF TIR domain-containing adaptor inducing interferon-β
Acknowledgements

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I would also like to express my great appreciation towards Dr. Leonard Foster, Dr. Clive Roberts and Dr. Hugh Kim for their active involvement in my thesis committee, spending precious time and providing invaluable expertise and direction for my research and thesis preparation.

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Dedication

To my parents and Todd Murray.
1 Introduction

1.1 Monoamine Oxidase

Monoamine oxidase (MAO) is a group of flavo-enzymes bound to the outer mitochondrial membrane that catalyze the oxidative deamination of monoamine hormones, neurotransmitters and dietary amines (Rodríguez et al., 2001; Binda et al., 2002; Youdim et al., 2006). MAO contains the redox cofactor, flavin adenine dinucleotide (FAD), which plays a central part in the oxidation of amine substrates. MAO is essential for brain development and function. Their involvement in the metabolism of neurotransmitters make MAO inhibitors (MAOIs) ideal for treating mood and neurodegenerative disorders (Binda et al., 2007).

1.1.1 MAO A and B

MAO A and MAO B are two isoforms of the MAO enzyme. They are present in most mammalian tissues (Youdim et al., 2006). They are encoded by two distinct but closely related genes on the X chromosome sharing an identical intron-exon structure, and a 72% overall sequence identity. The most conserved exon encoding the FAD-binding site are almost identical in MAO A and B (Binda et al., 2002; Grimsby et al., 1991; Carrieri et al., 2002; Youdim et al., 2006). Human MAO B crystallizes as dimers, whereas human MAO A crystallizes as monomers (Youdim et al., 2006). The overall tertiary structures of MAO A and MAO B are similar but the active sites are different, in that 7 out of the 20 residues are different in the substrate-recognizing region, leading to differences in conformation and substrate specificity (De Colibus et al., 2005). Both enzymes share the same catalytic mechanism because of their common FAD cofactor and substrate-oxidizing tyrosine composition in the active sites (De Colibus et al., 2005). They can
be distinguished based on substrate specificity and inhibition by synthetic compounds (Grimsby et al., 1991; Carrieri et al., 2002; Vindis et al., 2001; Youdim et al., 2006). MAO A preferentially degrades serotonin, MAO B has a greater affinity for phenylethylamine and benzylamine, and both isoforms share specificities for dopamine, epinephrine, norepinephrine, tryptamine and tyramine (Binda et al., 2002; Youdim et al., 2006). Interestingly, MAO A or B can also differ in substrate affinity within its own group in different types of tissue. In human for example, liver MAO B has affinity for imidazolines but platelet MAO B does not (Youdim et al., 2006).

1.1.2 Tissue Distribution in Human

MAO is present ubiquitously in mammalian tissues and the proportions between type A and B vary from tissue to tissue (Youdim et al., 2006). In human, MAO B expression varies in different tissue types but is found most abundant in the central nervous system, liver, kidney and small intestine (Rodríguez et al., 2001; Vindis et al., 2001; Inoue et al., 2015). It is also elevated in neuronal tissue with age (Binda et al., 2002; Youdim et al., 2006). In the human brain, type A and B are not evenly distributed. There are regional differences in brain MAO localization. Both A and B are highly expressed in the basal ganglia and hypothalamus, but less expressed in the cerebellum and cortex. Astrocytes and the basal ganglia contain higher levels of MAO B, whereas catecholaminergic neurons contain mostly MAO A (Youdim et al., 2006).

1.1.3 Biochemical Functions in Human

MAOs limit amine uptake by degrading dietary amines (Youdim et al., 2006). In peripheral tissues, MAO catabolize circulatory amines and control the entry of dietary amines. In the central nervous system, they protect neurons from toxic peripheral amines, terminate
neurotransmitter signals, and regulate intracellular amine levels (Abbott et al., 2006; Kaludercic et al., 2011). The overall MAO enzymatic reaction is shown below:

\[
RCH_2NR_1R_2 + O_2 + H_2O \overset{MAO}{\longrightarrow} RCHO + NR_1R_2 + H_2O_2
\]

MAO activity generates abundant reactive oxygen species (ROS) as byproducts, such as hydrogen peroxide, aldehyde and ammonia (Tipton et al., 2004). High levels of ROS are cytotoxic and sources of oxidative stress, which can induce tissue damage, inflammation and cancer (Vindis et al., 2001; Youdim et al., 2006; Kaludercic et al., 2011; Schieber and Chandel, 2014). MAO B is associated with the development of neurodegenerative disorders such as Parkinson’s and Alzheimer’s diseases. MAO B protein expression level is upregulated in glial cells and astrocytes in the brain of those patients. Subsequently, the excessive production of ROS is hypothesized to drive the neurodegenerative pathology seen in Parkinsonism (Binda et al., 2002; Youdim et al., 2006). Glial mitochondrial MAO B protein expression is also significantly upregulated in malignant glioma (Sharpe et al., 2015). MAO B in the myocardial mitochondria maintains normal cardiac function by regulating intracellular catecholamine levels (Kaludercic et al., 2014). In heart failure, MAO B enhances catecholamine degradation when concentration of circulatory catecholamines in the myocardium is increased. Increased levels of ROS produced by enhanced MAO B activity contributes to myocardial mitochondrial dysfunction and apoptosis (Kaludercic et al., 2011; Kaludercic et al., 2014). Increased levels of serum MAO show positive correlation with collagen synthesis in the liver and are associated with hepatic fibrosis in cirrhosis and chronic hepatitis (Hiroshi et al., 1978; Tsujii et al., 1977; Gressner, 1987).
1.1.4 MAO Inhibition

1.1.4.1 MAO A and B Selectivity

MAOIs were first developed as potent antidepressants following the discovery of MAO (Youdim et al., 2006). MAOIs are classified as selective, non-selective, reversible and irreversible forms (Yamada and Yasuhara, 2004). The early MAOIs developed were non-selective and irreversible (Youdim et al., 2006). Irreversible MAOIs covalently bind to the FAD cofactor of MAO to occupy its active site and prevent the enzymatic reaction (Youdim et al., 2006). However, due to their adverse side effects, selective reversible MAO A inhibitors and selective MAO B inhibitors were later developed to replace traditional MAOIs. MAO A is selectively inhibited by clorgyline irreversibly and by moclobemide reversibly, whereas MAO B is selectively and irreversibly inhibited by L-deprenyl, pargyline and rasagiline (Yamada and Yasuhara, 2004; Youdim et al., 2006).

1.1.4.2 Side Effects of MAO Inhibition

Nonselective and irreversible MAOIs are known for the risk of causing hypertensive crisis, also known as the “cheese effect” (Yamada and Yasuhara, 2004). MAO A in the intestine catalyzes the oxidation of dietary tyramines from fermented foods such as cheese, wine and cured meat (Carrieri et al., 2002). The blockage of tyramine degradation by nonselective and irreversible MAO inhibition causes tyramine accumulation in the circulation. Excessive tyramine uptake by adrenergic neurons can induce the release of noradrenaline, a neurotransmitter that raises the blood pressure, causing dangerous and even fatal hypertensive crisis (Youdim et al., 2006). Because the intestine contains little MAO B and it is responsible for metabolizing only a small portion of tyramine, tyramine degradation is not affected by selective irreversible MAO B
inhibition (Youdim et al., 2006). Therefore, selective MAO B inhibitors are preferred over classical MAOIs as safer alternatives due to their lack of the “cheese effect”. Selective and reversible MAO A inhibitors (RIMAs) such as moclobemide are also free of hypertensive crisis, thus have better safety profiles (Carrieri et al., 2002; Yamada and Yasuhara, 2004).

1.1.4.3 Clinical Applications in Mood and Degenerative Disorders

MAOIs are currently in clinical use for the treatment of psychiatric disorders and neurodegenerative diseases in the CNS, including depression, anxiety, Parkinson’s disease and Alzheimer’s disease (Youdim and Bakhle, 2006). These inhibitors are highly penetrative across the blood-brain barrier (BBB). The antidepressant properties of MAO inhibitors result from selective inhibition of MAO A and the accumulation of serotonin, dopamine, and noradrenaline in the brain (Yamada and Yasuhara, 2004; Youdim et al., 2006). Selective irreversible MAO B inhibition by L-deprenyl in conjunction with L-DOPA has proven effective in treating Parkinson’s disease. Patients with Parkinson’s disease are known to exhibit dopamine deficiency and lesions in the brain. Therefore, inhibiting MAOs as a mean of protecting dopamine levels and reducing the levels of ROS from amine oxidation helps slow down dopamine degradation and reduce brain tissue damage (Youdim et al., 2006; Youdim and Bakhle, 2006).

1.1.4.4 Effects in Non-CNS Tissues

MAO inhibitors have also shown potential therapeutic value in treating non-CNS chronic inflammation. In 1998, Dr. Kast reported a case of improved chronic inflammatory condition in a patient with Crohn’s disease and depression who was prescribed a nonselective MAO inhibitor, phenelzine, as an antidepressant (Kast, 1998; Youdim et al., 2006). In a rat periodontal disease
study, oral mucosal inflammation was induced by bacterial lipopolysaccharide, MAO B gene was significantly upregulated in the inflamed epithelium, and MAO B protein expression was increased 6-fold in the diseased tissues compared to healthy control (Ekuni et al., 2009). Nonselective inhibition of MAO by topical application of phenelzine reduced characteristics associated with periodontal disease, such as periodontal epithelial migration, alveolar bone loss, polymorphonuclear leukocyte infiltration and increased level of serum H₂O₂ (Ekuni et al., 2009). Selective irreversible MAO B inhibition by L-deprenyl or pargyline demonstrated protective effects in animal models of cardiac failure, cerebral ischemia and renal ischemia-reperfusion (Kunduzova et al., 2002; Chaaya et al., 2010; Kaludercic et al., 2011). MAO B knockout and pargyline treatment also prevented pathological changes such as left ventricular dilation and fibrosis in induced cardiac failure in mice (Kaludercic et al., 2014). Most of these studies showed that high levels of MAO-derived H₂O₂ production was associated with disease and reduced by MAO inhibition, suggesting that reduction of ROS by MAO inhibition may have therapeutic potentials not only in the CNS but also in the non-CNS peripheral tissues.

1.1.4.5 Development of Novel MAO B Inhibitors

Despite its strong potency and MAO B selectivity, deprenyl is metabolized into potentially addictive, mood-stimulating amphetamines (Tabakman et al., 2004; Lecht et al., 2007; Bolea et al., 2013). In recent years, more novel MAO B inhibitors have been developed to improve the current pharmacological profile for the treatment of Parkinson’s and Alzheimer’s disease, such as imidazolium salts, benzylxynitrostyrene derivatives, and propargylamine-derived compounds like rasagiline and PF9601N (Lecht et al., 2007; Unzeta and Sanz, 2011; Bolea et al., 2013; Huleatt et al., 2015; Van der Walt et al., 2017; Chan et al., 2018). Novel reversible MAO B
inhibitors are also synthesized as tracer compounds to radiolabel MAO B in the brain for \textit{in vivo} molecular imaging in the diagnosis of Alzheimer’s disease (Neudorfer et al., 2014). High BBB permeability is required for these compounds to enter the brain and target the CNS.

1.2 Novel MAO B Inhibitors with Reduced BBB Permeability

Dr. Edward Putnins collaborated with the Centre for Drug Research and Development and Dr. David Grierson from the Faculty of Pharmaceutical Sciences at the University of British Colombia to synthesize novel selective reversible MAO B inhibitors with reduced brain permeability. The BBB (Figure 1.1) is composed of the endothelium lining the luminal surface of cerebral capillaries that separates the interstitial fluid in the brain from the peripheral circulation (Abbott et al., 2006). It regulates the homeostasis of the neuronal microenvironment and prevent fluctuations in interstitial fluid composition by controlling the ionic and molecular exchange between the blood and the brain. The paracellular access between adjacent BBB endothelial cells are sealed by a complex network of tight junctions, so that most ions and molecules must undergo highly selective transcellular transportations mediated by receptors, channels and transport proteins to enter the brain (Abbott et al., 2006). The lipid bilayer structure and the tight junctions of the BBB allow free diffusion of small lipophilic drugs but restrict the entry of polar and hydrophilic drugs (Scherrmann, 2002). Therefore, the BBB permeability of the novel inhibitors can be reduced by increasing their polarity and hydrophilicity.
**Figure 1.1 The blood-brain barrier**

The BBB is the endothelium of the cerebral microvasculature, supported by astrocyte foot processes. The barrier features of BBB endothelial cells, such as the formation of tight junctions, are induced by factors released from the surrounding astrocyte foot processes. From “Blood-Brain Barrier”, by J. Perkins. Copyright 2002 by Elsevier Inc. Reprinted with permission.

Based on the structure of deprenyl (Figure 1.2) as the parent compound, 50 novel compounds were synthesized. The structure of deprenyl can be divided into three zones. Each novel compound bears unique structural modifications on one or more of the three zones, which make them more lipid-insoluble than deprenyl. The compounds were screened to select those with specificity for MAO B over MAO A, low IC\textsubscript{50}’s of MAO B, and low BBB permeability in vitro and in vivo.
Figure 1.2 The structure of deprenyl
The novel MAO B inhibitors are structurally analogous to deprenyl, with structural modifications that make them more polar and hydrophilic than deprenyl. Copyright by CDRD. Reprinted with permission.

1.2.1 Characteristics

Four lead compounds were selected from the initial screening. They were designated as Compound A, B, C and D. They all exhibited specificities towards MAO B 100-fold over MAO A, IC₅₀’s of MAO B lower than 0.3 µM (Figure 1.3) and reduced BBB permeability (Figure 1.4) (Gealageas et al., 2018). Low IC₅₀’s of MAO B and high IC₅₀’s of MAO A indicated that the compounds were far more selective towards MAO B than MAO A (Figure 1.3). The BBB permeability in vivo was measured as brain versus plasma concentration ratio in mice via intravenous administration, and compared to that of their parent compound, deprenyl (Figure 1.4). In the plasma, deprenyl reached a maximal concentration (C_max) of only less than 600 ng/mL, whereas novel compounds showed much higher plasma concentrations (C_max).
Conversely, in the brain, deprenyl reached a higher brain versus plasma concentration ratio shown as K_p (AUC_last brain/plasma), indicating that deprenyl is strongly brain-permeable. In contrast, novel compounds showed reduced brain concentrations (C_max), resulting in much lower brain versus plasma concentration ratios (Gealageas et al., 2018). The lead compounds showed
much lower BBB permeability than that of deprenyl, and were well absorbed via intravenous, subcutaneous and oral administration routes (Gealageas et al., 2018).

Figure 1.3 IC$_{50}$'s of novel MAO B inhibitors
Concentrations of novel MAO B inhibitors, Compound A, B, C and D, producing 50% inhibition of MAO A (upper) and MAO B (lower) enzymes expressed as IC$_{50}$’s. Copyright 2018 by American Chemical Society: Journal of Medicinal Chemistry, Gealageas et al. Reprinted with permission.
Figure 1.4 Reduced BBB permeability of novel MAO B inhibitors
Mice plasma (upper) and brain (lower) pharmacokinetic parameters of intravenously administrated novel MAO B inhibitors, Compound A, B, C and D, in comparison to their parent compound, deprenyl. BBB permeability was measured by $K_p$ ($\text{AUC}_{\text{last}}$ brain/plasma). Copyright 2018 by American Chemical Society: *Journal of Medicinal Chemistry*, Gealageas et al. Reprinted with permission.

1.2.2 Anti-inflammatory Effects

Lipopolysaccharide (LPS) is a bacterial endotoxin found on the outer membrane of Gram-negative bacteria. It is a virulent bacterial antigen that can induce inflammatory response both *in vitro* and *in vivo*. LPS from *E. coli* induced signs of periodontal disease in rat (Ekuni et al., 2009). In a recent study by the Putnins lab, *E. coli*-derived LPS has also been shown to upregulate cytokine gene and protein expression in human intestinal endothelial and epithelial cell models. In the intestinal cell models, Compound B, C and D demonstrated effectiveness at reducing the levels of LPS-induced cytokine and chemokine gene expression and secretion (Tra, 2015).
1.2.2.1 Cytokine Expression in Intestinal Endothelium and Epithelium

The intestinal mucosal epithelium functions as a barrier between the lumen and the submucosa, providing the first line of defense against intestinal bacterial microflora (Heidemann et al., 2006; Vaure and Liu, 2014). Commensal Gram-negative bacteria carry virulent microbial antigens, such as LPS, lipoprotein and flagellin. Exposure to bacterial antigens compromises the barrier integrity of the epithelium. In response, epithelial cells express cytokines and chemokines to recruit local immune cells and induce a localized immune response. Both cancerous and primary human intestinal epithelial cells express cytokines and chemokines such as interleukin-6 (IL-6), interleukin-8 (IL-8), monocyte chemoattractant protein-1 (MCP-1), granulocyte-macrophage colony-stimulating factor (GM-CSF) and TNF-α (tumor necrosis factor-α) in response to invasive gram-negative bacteria (Jung et al., 1995; Stadnyk, 2002). Interestingly, many reports showed that some of these cells, such as the T84 and Caco-2 cell lines, responded poorly to LPS stimulation (Jung et al., 1995).

The microvascular endothelium is the internal lining of blood and lymph vessels made up of a monolayer of endothelial cells (Cibor et al., 2016). It functions as a physical and regulatory barrier between the circulation and tissue submucosa, and plays an important role in innate immunity. Healthy endothelium expresses a wide array of peptides and factors regulating vascular tension, angiogenesis, blood coagulation and haematopoiesis (Cibor et al., 2016). When pathogens leak through the disrupted epithelium into the submucosal microvasculature (Figure 1.5), endothelial cells express pro-inflammatory cytokines, chemokines, cellular adhesion molecules and growth factors in response, which recruit circulating immune cells to sites of inflammation, increase endothelial permeability and induce angiogenesis, leading to an aggravated immune response (Heidemann et al., 2006; Vaure and Liu, 2014; Cromer et al., 2011;
Cibor et al., 2016). Current understanding of human intestinal endothelium is established largely based on studies done on cultured primary human intestinal microvascular endothelial cells (HIMEC). Stimulation with LPS induces the expression of IL-1α, IL-6, IL-8, TNF-α and GM-CSF in HIMEC cells (Nilsen et al., 1998).

**Figure 1.5 Bacterial invasion in the intestinal mucosa**
Luminal bacterial invasion comprises the integrity of the intestinal epithelium. Epithelial cells express cytokines to recruit local leukocytes to eliminate bacteria in the lamina propria. Endothelial cells, in reaction to the local immune response, express cytokines and adhesion molecules to induce infiltration of circulating leukocytes into the lamina propria to join the defense. The added effect of local and distant immune responses lead to exacerbated inflammation. Copyright 2006 by American Society for Microbiology: *Infection and immunity*, Heidemann et al. Reprinted with permission.

Previously, the Putnins lab used HIMEC as the endothelial cell model, and the cancerous human intestinal epithelial cell line Caco-2 as the epithelial cell model, to study mucosal response to LPS in terms of cytokine expression (Tra, 2015). Stimulation with LPS from *E. coli* serotype O55:B5 significantly increased the secretion of IL-10, interferon-γ (IFN-γ), IL-12p70,
IL-1β, TNF-α, IL-6 and IL-8 in HIMEC cells over 12 hours. In undifferentiated Caco-2 cells, LPS increased the levels of IL-12p70, IL-6, IL-8 and IL-10 secretion (Tra, 2015). LPS stimulation also significantly upregulated the mRNA expression of IL-6, IL-8 and TNF-α in HIMEC cells, and the mRNA expression of IL-8 in undifferentiated Caco-2 cells (Tra, 2015). These results indicate that the cell models used are responsive to LPS stimulation in terms of cytokine gene and protein expression.

1.2.2.2 Reduction of Cytokine Expression in Intestinal Endothelial and Epithelial Cell Models by Novel MAO B Inhibitors

Excess cytokine signaling leads to prolonged immune response and aggravation of inflammation. Therapeutics designed to inhibit cytokine expression or inactivate secreted cytokines may reduce inflammation. The Putnins lab showed that Compound B, C and D reduced the levels of LPS-induced IL-8 gene and protein expression in Caco-2 cells, and the levels of LPS-induced gene and protein expression of IL-8, IL-6 and TNF-α in HIMEC cells (Tra, 2015). Especially, Compound B showed the highest efficacy. It is hypothesized that novel compounds may impose their downregulating effects by disrupting one or more of the LPS-activated intracellular signaling pathways that regulate cytokine gene and protein expression. The common signaling pathways regulating cytokine expression activated by LPS include the NF-κB and MAPKs pathways downstream of Toll-like receptor (TLR) signal transduction (Gay et al., 2014).
1.3 LPS/TLR Signaling Pathways

1.3.1 Toll-like Receptors

Microbial surface ligands, such as LPS, and viral genetic materials are pathogen-associated molecular patterns (PAMPs). Toll-like receptors (TLRs) are membrane receptors that can activate innate immunity through the recognition of pathogen-associated molecular patterns (PAMPs) (Takeda and Akira, 2005; Lu et al., 2008). The human TLR family consists of ten members found on the cell surface or the endosome (Takeda and Akira, 2005; O’neill et al., 2013). The Toll-like receptor 4 (TLR4) is the primary receptor that recognizes LPS, and it is located on the cell surface (Takeda and Akira, 2005; O’neill et al., 2013; Vaure and Liu, 2014). The Toll-like receptor 2 (TLR2), another member of the cell surface TLRs, has also been reported to recognize certain LPS with non-classical structures (Takeda and Akira, 2005).

1.3.1.1 TLR4

TLR4 is only functional when formed as a complex. The TLR4 complex (Figure 1.6) consists of homodimeric TLR4, membrane-anchored co-receptor cluster of differentiation 14 (CD14), myeloid differentiation factor 2 (MD-2), and soluble LPS binding protein (LBP) on the extracellular face of the plasma membrane (Finlay and Hancock, 2004; Lu et al., 2008; Vaure and Liu, 2014). On the cytoplasmic side, the Toll/IL-1R (TIR) domains on TLR4 recruit multiple adaptor proteins upon TLR4 activation, including MyD88 adaptor-like protein a.k.a. TIR domain-containing adaptor protein (Mal/TIRAP), myeloid differentiation primary response protein 88 (MyD88), TIR domain-containing adaptor inducing interferon-β (TRIF), and TRIF-related adaptor molecule (TRAM) (Figure 1.6). Mal/TIRAP and MyD88 mediate the MyD88-dependent pathway that regulates the gene expression of pro-inflammatory cytokines and
chemokines, whereas TRAM and TRIF mediate the MyD88-independent pathway regulating the
gene induction of type I interferons (Takeda and Akira, 2005; Lu et al., 2008; Ofengeim and
Yuan, 2013; Gay et al., 2014; Vaure and Liu, 2014). Studies have shown that responsiveness to
LPS requires the expression of all the co-receptors and cytoplasmic adaptor proteins in the cell
(Abreu et al., 2001; Funda et al., 2001; Naik et al., 2001; Böcker et al., 2003; Tyrer et al., 2006;
Lenoir et al., 2008; Hsu et al., 2011; Kuo et al., 2015). In human, TLR4 is expressed most
abundantly in monocytes, macrophages, and lymphocytes found in the peripheral blood and
spleen (Vaure and Liu, 2014). TLR4 is also detected in glial cells, colon, small intestine, ovary
and placenta. TLR4 upregulation has been observed upon LPS stimulation and was found to be
associated with various diseases, such as inflammatory bowel disease, heart failure and multiple
sclerosis (Vaure and Liu, 2014).
Figure 1.6 The TLR4 complex and its adaptor protein-mediated pathways
Besides homodimeric TLR4, the TLR4 complex also contains co-receptors including membrane CD14 and MD-2. LPS activation of pro-inflammatory cytokine expression requires mediation by TIRAP and MyD88. LPS activation of type I interferons requires mediation by TRAM and TRIF. Copyright 2008 by Elsevier Ltd.: Cytokine, Lu et al. Reprinted with permission.

1.3.1.2 TLR2

TLR2 forms heterodimers with TLR1 or TLR6 to build functional TLR2 receptor complexes on the cell surface that recognize bacterial triacyl- and diacyl-lipopeptides (Takeda and Akira, 2005; O'Neill et al., 2013). TLR2 receptors also respond to a variety of other microbial ligands such as LPS from certain bacteria, peptidoglycan, glycolipids, and glucans found on the fungal cell wall (Takeda and Akira, 2005). This versatility is attributed to their heterodimeric structures. TLR2 receptors were shown to be reactive towards atypically structured LPS from non-enterobacteria species such as *Leptospira interrogans*, *Porphyromonas gingivalis*, and *Bacteroides fragilis* (Medzhitov, 2001; Alhawi et al., 2009). Some research,
however, has shown that TLR2 activation in response to LPS is due to lipoprotein contamination from inefficient purification procedure (Abreu et al., 2001; Takeda and Akira, 2005).

1.3.2 Intracellular Signaling Pathways Mediating LPS-induced IL-8 Gene Expression

In the LPS-induced TLR4-mediated signaling (Figure 1.7), soluble LBP present extracellular LPS to CD14 on the cell surface, where LPS is recognized by the TLR4 complex. LPS recognition by TLR4 triggers a signaling cascade mediated by interleukin-1 receptor associated kinases (IRAKs) and tumor necrosis factor receptor associated factor 6 (TRAF6) in the cytoplasm. IRAKs and TRAF6 activate downstream kinases, including the TGF-β-activated kinase 1 (TAK1) complex, mitogen-activated protein kinase kinases kinase 1 (MEKK1) and the IκB kinase (IKK) complex. These kinases further activate downstream mitogen-activated protein kinases (MAPKs) and transcription factor nuclear factor kappa-B (NF-κB), which induce gene transcription of pro-inflammatory cytokines and chemokines in the nucleus (Finlay and Hancock, 2004; Takeda and Akira, 2005; Viatour et al., 2005; Lu et al., 2008; Abreu, 2010; Arthur and Ley, 2013; Ofengeim and Yuan, 2013; Kawasaki and Kawai, 2014; Gay et al., 2014). This signaling pathway is present in intestinal epithelial cells, lung epithelial and endothelial cells, fibroblasts, and innate immune cells such as dendritic cells and macrophages (Abreu, 2010; Arthur and Ley, 2013; Kawasaki and Kawai, 2014; Vauré and Liu, 2014).
1.3.2.1 IL-8

IL-8 is a pro-inflammatory chemokine upregulated by LPS and cytokine stimulation in both immune and non-immune cells (Harada et al., 1994; Hoffmann et al., 2002). IL-8 is a specific chemoattractant for neutrophils and T-lymphocytes (Schuerer-Maly et al., 1994). IL-8 induces neutrophil and T-cell activation, migration and infiltration into sites of inflammation, thus causing subsequent tissue damage (Harada et al., 1994). Activated neutrophils release granular enzymes that can degrade connective tissue (Bickel, 1993). Augmented levels of
chemokine production and neutrophil infiltration in tissues are characteristic of inflammation (Baggiolini and Clark-Lewis, 1992). The IL-8 promoter sequence (Figure 1.8) contains binding sites for its transcription factors NF-κB, activator protein-1 (AP-1) and nuclear factor for interleukin-6 (NF-IL-6) (Roebuck, 1999; Hoffmann et al., 2002). LPS-induced IL-8 gene expression is regulated by NF-κB and ERK, p38 and JNK MAPKs across different cell types, including the endothelium, epithelium, fibroblasts, mononuclear and polymorphonuclear leukocytes (Hoffmann et al., 2002).

![Image of IL-8 promoter](image)

**Figure 1.8 The IL-8 promoter**
The IL-8 promoter contains binding sites for transcription factor AP-1, C/EBP and NF-κB. Copyright 2002 by the Society for Leukocyte Biology: Journal of Leukocyte Biology, Hoffmann et al. Reprinted with Permission.

### 1.3.2.2 NF-κB Pathway

The p50/p65 (RelA) NF-κB is a heterodimeric transcription factor complex that plays a critical role in the induction of inflammation (Lawrence, 2009). In resting cells, p50/RelA NF-κB is bound to its inhibitory molecule, IκB, which keeps NF-κB inactive; upon microbial or cytokine stimulation, activation of NF-κB requires the phosphorylation and subsequent ubiquitination of IκB by the IKK complex to release NF-κB enabling its translocation from the cytoplasm into the nucleus, and subsequent induction of gene expression (Figure 1.9) (Viatour et al., 2005; Vermeulen et al., 2006; Christian et al., 2016). Optimal induction of p50/RelA NF-κB
transcriptional activity is also regulated through phosphorylation and dephosphorylation at various serine and threonine sites on the RelA subunit (Figure 1.10) (Viatour et al., 2005; Vermeulen et al., 2006; Christian et al., 2016; Chen and Greene, 2004). These events occur both in the cytoplasm and nucleus. Therefore, phosphorylation and nuclear translocation are common parameters assessed in most studies to determine NF-κB activation.

Figure 1.9 Activation of the NF-κB pathway by LPS via TLR
Figure 1.10 Regulation of NF-κB activity by phosphorylation of RelA/NF-κB p65

1.3.2.3 MAPKs Pathways

MAPKs belong to a versatile family of kinases found in eukaryotic cells (Roux and Blenis, 2004). In response to extracellular stimuli such as growth factors, stress factors and pro-inflammatory cytokines, MAPKs are activated by MAPK kinases (MAPKKs/MEKs) via dual phosphorylation at threonine and tyrosine residues in a conserved activation region. Phosphorylated MAPKs then translocate from the cytoplasm into the nucleus, and phosphorylate
a wide variety of downstream substrates, such as transcription factors and protein kinases, which carry out important cellular functions ranging from gene expression, proliferation, mitosis and differentiation to apoptosis (Roux and Blenis, 2004). The three most well characterized groups of MAPKs are the extracellular signal-regulated kinases 1 and 2 (ERK1/2), c-Jun N-terminal kinase (JNK) and p38 kinases. In general, ERK1/2 are activated by growth factors, whereas p38 and JNKs are activated by environmental stress and pro-inflammatory mediators, such as bacterial and viral infection, cytokines, chemokines and LPS (Roux and Blenis, 2004). During the regulation of TLR-mediated IL-8 gene expression (Figure 1.11), phosphorylated ERK1/2 and JNK activate the two subunits of transcription factor AP-1, c-Fos and c-Jun, respectively (Karin, 1995; Hoffmann et al., 2002; Roux and Blenis, 2004). Activated p38 regulates a series of important events in immune and inflammatory responses, such as neutrophil migration, cytokine and chemokine mRNA stabilization, translation and exocytosis, including those of IL-8 (Hoffmann et al., 2002; Roux and Blenis, 2004). When phosphorylated, p38 directly activates c-AMP-responsive element-binding protein (CREB), a transcription factor for cytokine and chemokine gene expression during inflammation (Gay et al., 2014). The p38 MAPK pathway also regulates actin reorganization, a process required during cytokine and chemokine exocytosis, thus p38 may also be involved in the vesicular export mechanism of IL-8 protein secretion (Roux and Blenis, 2004; Stanley and Lacy, 2010; Shandala and Brooks, 2012).
Besides the transcription factors mentioned above, CCAAT-enhancer-binding protein-β (C/EBP-β), also known as nuclear factor for IL-6 expression (NF-IL-6), also regulates IL-8 gene transcription (Akira et al., 1990; Mukaida et al., 1994; Roebuck, 1999; Hoffmann et al., 2002). NF-IL-6 mRNA expression is induced by LPS stimulation. Though NF-κB is essential, NF-IL-6
and AP-1 function in coordination with NF-κB in the induction of IL-8 gene expression. In addition, research by Sadeghi et al. showed that reduced IL-8 production was associated with reduced expression of interferon regulatory factor 5 (IRF-5), MyD88 and TLR4 in monocytes, although there is no sufficient evidence indicating that IRF-5 directly regulates IL-8 expression (Sadeghi et al., 2007). IRF-5 is a well-known transcription regulator for LPS/TLR4-induced IL-6, TNF-α, and IL-12 expression (Takaoka et al., 2005).
1.4 Rationale, Objectives and Hypothesis of this Study

**Rationale:** LPS induced IL-8 gene and protein expression, which were downregulated by novel MAO B inhibitors Compound B, C and D in undifferentiated Caco-2 cells (Tra, 2015). Especially, Compound B was shown to be the most effective. However, the mechanism of induction of IL-8 gene and protein expression by LPS and the mechanism of their downregulation by Compound B are unknown. According to the literature, LPS typically signals through NF-κB- and MAPK-mediated signaling pathways to induce IL-8 gene and protein expression, such as NF-κB nuclear translocation, phosphorylation of NF-κB p65 at Ser536 and phosphorylation of ERK1/2, p38 and JNK MAPKs. Therefore, we begin to investigate the potential effects of LPS and Compound B on NF-κB- and MAPK-mediated signaling.

**Hypothesis:** Novel MAO B inhibitor Compound B reduces LPS-induced IL-8 gene and protein expression in Caco-2 cells by inhibiting NF-κB- and/or MAPK-mediated signaling.

**Global Aim:** Examine the classical signaling pathways regulating LPS-induced IL-8 gene and protein expression in Caco-2 cells to determine if they are downregulated by the novel MAO B inhibitor Compound B.

**Specific Aims:**

1) Examine the effect of Compound B on LPS-induced NF-κB signaling in Caco-2 cells via the following:
   a. NF-κB p65 Ser536 phosphorylation using Western blotting
   b. NF-κB p65 nuclear translocation using immunofluorescence staining
2) Examine the effect of Compound B on LPS-induced MAPK signaling in Caco-2 cells via the following:

   a. ERK1/2 Thr202/Tyr204 phosphorylation using Western blotting
   b. p38 Thr180/Tyr182 phosphorylation using Western blotting
   c. JNK Thr183/Tyr185 phosphorylation using Western blotting
2 Materials and Methods

2.1 Materials

The Caco-2 cell line (ATCC® HTB-37™) and the HepG2 cell line (ATCC® HB-8065™) were generously provided by CDRD. Raw264.7 cell line (ATCC® TIB-71™) was a kind gift from the Overall lab at UBC. Fetal bovine serum (cat. #12483-020), DMEM (cat. #10313-021), MEM Alpha (cat. #12561-056), PBS (cat. #10010-049), 0.25% trypsin-EDTA (cat. #25200-072), GlutaMAX™ supplement (cat. #35050061), 10,000 U/mL penicillin-streptomycin (cat. #15140122), and trypan blue (cat. #15250-061) were from Gibco® by Life Technologies. DMEM (cat. #D6429) was from Sigma-Aldrich. 75 cm² (T75) tissue culture-treated flasks (cat. #83.3911.002), 2-position blade soft rubber cell scrapers (cat. #83.1830), and 15 mL sterile conical tubes (cat. #62.554.205) were from Sarstedt. Microcentrifuge tubes 0.6 mL (cat. #MCT-060-C) and 1.7 mL (cat. #MCT-175-C) were from Axygen. Falcon® Multiwell™ 6-well tissue culture plate (cat. #353046), Blue Max™ 50 mL sterile conical tubes (cat. #352098), 96-well non-tissue culture-treated flexible assay microplates (cat. #353912), and Falcon® Cyclopore® 0.45 µm pore PET membrane 25 mm ø cell culture inserts (cat. #3090) (Figure 2.1) were from Becton Dickinson Labware. Costar® 96-well flat clear bottom black tissue culture-treated microplates (cat. #3603), 24-well clear tissue culture-treated plates (cat. #3524), and Transwell® 0.4 µm pore polyester membrane 6.5 mm ø cell culture inserts in 24-well tissue culture-treated plates (cat. #3470) (Figure 2.1) were from Corning Inc.
Figure 2.1 Caco-2 cell differentiation in Transwell insert
Caco-2 cell differentiation is induced by growing cells in 6.5 mm ø or 25 mm ø Transwell inserts with porous polyester (PET) membrane. Cells are seeded on the porous membrane in the apical media compartment. Porous membranes are 0.4 – 0.45 µm ø in pore size. Adapted from Corning Inc. Copyright 1994-2018 by Corning Inc.

In various assays, LPS from *Escherichia coli* serotype O55:B5 (cat. #L2880-25MG), deprenyl (cat. #M003), PD98059 (cat. #P215), bovine serum albumin (cat. #A7906-100G), mouse IgG from serum (cat. #I5381), normal goat serum (cat. #G9023), recombinant human MAO B (cat. #M7441) and poly-D-lysine hydrobromide (cat. #P0899) were from Sigma-Aldrich. Human recombinant IL-1β (cat. #CYT-208) was from ProSpec. Type 1 ultrapure H₂O (18.2 MΩ/cm) was purified using Milli-Q® Integral water purification system from EMD Millipore. Human IL-8 tissue culture kits (cat. #K151ANB-1) and SECTOR S 600 instrument were from MSD. Novex™ Dynabeads® Protein G (cat. #10004D), Invitrogen™ Dynal® DynaMag™-2 magnet (cat. #123.21D), ProLong™ Gold Antifade reagent with DAPI (cat. #36931), and Molecular Probes® Alexa Fluor 488 goat anti-mouse IgG secondary antibody (cat. #A11001) were from Invitrogen by Life Technologies. Anti-mouse/human RelA/NF-κB p65 (Clone #532301) mouse monoclonal antibody (cat. #MAB5078) was from R&D Systems.
For Western blotting, RIPA lysis buffer system with PMSF, sodium orthovanadate and protease inhibitor cocktail (cat. #sc-24948) was from Santa Cruz Biotechnology. Bimake® protease inhibitor cocktail (cat. #B14002) and phosphatase inhibitor cocktail (cat. #B15002) were from Bimake. Omega bio-tek® homogenizer mini columns (cat. #HCR003) was from Omega Bio-tek Inc. QIAshredder spin columns (cat. #79654) was from QIAGEN. Pierce® BCA Protein Assay Kit (cat. #23225), PageRuler™ pre-stained protein marker (cat. #26616), and Restore™ Fluorescent Western blot stripping buffer (cat. #62299) were from Thermo Fisher Scientific. Blok™ – FL fluorescent blocker (cat. #WBAVDFL01) and Immobilon-FL transfer membrane (cat. #IPFL00010) were from EMD Millipore. Anti-MAO B Picoband™ rabbit polyclonal antibody (cat. #PB9665) was from Boster. Anti-MAO B (D-6) mouse monoclonal antibody (cat. #sc-515354), anti-NF-κB p65 (C-20)-G goat polyclonal antibody (cat. #sc-372-G), anti-JNK1 (FL) rabbit polyclonal antibody (cat. #sc-571) were from Santa Cruz Biotechnology. Anti-phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204) rabbit polyclonal antibody (cat. #9101), anti-p44/42 MAPK (Erk1/2) (137F5) rabbit monoclonal antibody (cat. #4695), anti-p38 MAPK (L53F8) mouse monoclonal antibody (cat. #9228S), and anti-phospho-NF-κB p65 S536 (93H1) rabbit monoclonal antibody (cat. #3033) were from Cell Signaling Technology. Anti-ACTIVE® p38 (pTGPpY) rabbit polyclonal antibody (cat. #V1211) and anti-ACTIVE® JNK (pTPpY) rabbit polyclonal antibody (cat. #V7931) were from Promega. Donkey anti-rabbit IgG IR Dye 800CW secondary antibody (cat. #926-32213), donkey anti-mouse IgG IR Dye 680RD secondary antibody (cat. #926-68072), and donkey anti-goat IgG IR Dye 680RD secondary antibody (cat. #926-68074) were from LI-COR Biosciences.
2.2 Cell Culture Experiments

Caco-2 cells between passage 31-50 were maintained in DMEM containing 10% FBS, 1% GlutaMax and 1% penicillin-streptomycin. HepG2 cells between passage 6-12 were cultured in MEM Alpha containing 10% FBS and 1% penicillin-streptomycin. Raw264.7 cells were cultured in DMEM containing 10% FBS and 1% penicillin-streptomycin. All cells were maintained on T75 TC-treated flasks at 37°C in a humidified incubator containing 5% CO₂. Caco-2 and HepG2 cells were passaged every 5 days until 90% confluent. Raw264.7 cells were passaged every 2-3 days until 90% confluent. To passage or seed Caco-2 or HepG2 cells, cells were washed once with 10 mL of sterile PBS pH 7.4, incubated with 2 mL of 0.25% Trypsin-EDTA for 5 min at 37°C in a humidified incubator containing 5% CO₂, neutralized with 4 mL of fresh culture media containing 10% FBS, transferred to clean 15 mL conical tubes (Sarstedt, Nümbrecht, Germany), centrifuged to pellet and resuspended in fresh culture media by repeated pipetting. An aliquot of cells was then passaged onto a new T75 flask, or stained with trypan blue, counted using a hemocytometer to calculate the number of cells per milliliter and seeded in various culturewares at the appropriate cell densities (see below). For passaging or seeding Raw264.7 cells, cells were washed once with 10 mL of sterile PBS pH 7.4, scraped off in 6 mL of fresh culture media with a cell scraper, resuspended, and seeded at a 1:3 ratio onto a new T75 flask for cell propagation, or counted and seeded in various culturewares at the appropriate cell densities (see below).

To induce Caco-2 cell differentiation, cells were seeded in 6-well plates at 3.54×10⁵ cells/1.5 mL/insert in the apical compartment in 25 mm ø inserts (Corning Inc., Corning, NY) (Figure 2.1) submerged in 2.6 mL of basal culture media per well, cultured for 2 days to reach confluency, and cultured for an additional 7 or 14 days after reaching confluence (Figure 2.2).
Fresh media was changed every other day. On Day 14, cells were harvested for the examination of MAO B expression via Western blotting (Figure 2.2).

Additionally, Caco-2 cells were seeded in 24-well plates at $2.5 \times 10^4$ cells/0.1 mL/insert/well in 6.5 mm Ø inserts (Corning Inc., Corning, NY) (Figure 2.1) submerged in 0.6 mL of basal culture media per well, cultured for 4 days to reach confluency, and cultured for an additional 14 days after reaching confluency (Figure 2.2). Fresh media was changed every other day. On Day 14, cells were subjected to LPS stimulation and drug treatments; on Day 15, cells were assayed for IL-8 secretion via ELISA (Figure 2.2).
Figure 2.2 Schematic diagram of the timeline of Caco-2 cell experiments
The timeline of experiments performed with undifferentiated Caco-2 cells is shown in (a) and the timeline of experiments performed with differentiated Caco-2 cells is shown in (b).
2.3 Cell Lysis

To harvest HepG2 and Caco-2 whole cell lysates, cells grown on T75 flasks were washed once with sterile PBS pH 7.4, and incubated with 0.25% trypsin-EDTA at 37°C in a humidified incubator containing 5% CO₂ for 5 min. Differentiated Caco-2 cells grown in Transwell inserts were strongly adherent and required at least 15 min of trypsinization. PBS and trypsin-EDTA were added to both the apical and basal compartment to ensure thorough wash and effective trypsinization. Cell suspension was then neutralized with fresh culture media containing 10% FBS, transferred to clean 15 mL conical tubes and centrifuged to pellet, transferred to clean 1.7 mL microcentrifuge tubes and washed twice with ice-cold PBS to eliminate remaining serum, and centrifuged for 5 min at 1600 rpm at 4°C on benchtop centrifuge 5424R (Eppendorf, Hamburg, Germany) after each wash to pellet the cells. The cells were resuspended in RIPA lysis buffer with freshly added protease inhibitor cocktail, PMSF and sodium orthovanadate at a ratio of 1:100 and incubated for 30 min on ice. After lysis, the whole cell lysates were centrifuged at 15000 rpm at 4°C for 10 min to separate the soluble and the insoluble fractions. The supernatants containing soluble proteins were transferred to fresh microcentrifuge tubes and either used immediately or stored at -80°C for future use. The insoluble pellets were discarded. Protein concentrations of whole cell lysates were determined using Pierce® BCA protein assay kit (Thermo Fisher Scientific, Waltham, MA) according to the manufacturer’s instruction manual.

To harvest Caco-2 and Raw264.7 whole cell lysates for the study of NF-κB and MAPK signaling pathways via Western blotting, cell culture media were discarded upon completion of treatments, the cells were washed once with ice-cold PBS pH 7.4, and lysed directly in the plate or inserts on ice with 50-200 µL of 1× Laemmli lysis buffer (4× Laemmli buffer diluted with PBS, pH 7.4 at a ratio of 1:3) (4× Laemmli buffer: 40% glycerol, 8% SDS, 250 mM Tris/HCl
pH 6.8, 0.04% bromophenol blue and 8% β-mercaptoethanol) with protease and phosphatase inhibitor cocktails (Bimake, Houston, TX) added immediately before use. Whole cell lysates were collected by scraping and pipetting, transferred into clean homogenizer mini columns (Omega Bio-tek, Norcross, GA) or QIAshredder spin columns (QIAGEN, Hilden, Germany), centrifuged at 15000 rpm for 3 min to homogenize, transferred to clean 1.7 mL microcentrifuge tubes, boiled for 5 min to denature and immediately used for Western blotting or stored at -80°C for future use.

2.4 Immunoprecipitation Anti-MAO B

Protein G-coupled Dynabeads were vortexed for 60 sec. to resuspend the beads and an aliquot of 50 µL (1.5 mg) per IP was transferred to a 1.7 mL microcentrifuge tube. The tube was placed on the magnet, the supernatant was removed. 5 µg of rabbit polyclonal Picoband™ anti-MAO B antibody [500 µg/mL] (Boster, Pleasanton, CA) was diluted in 200 µL wash buffer (PBS pH 7.4, 0.02% Tween-20) and added to the Dynabeads, resuspended and incubated with rotation at 4°C for 4 h. Fresh whole cell lysates of 90% confluent HepG2 and undifferentiated Caco-2 cells from T75 TC-treated flasks were harvested in RIPA lysis buffer as described in Section 2.3. After pre-incubation with antibody, Dynabeads were placed on the magnet and supernatant was removed. An aliquot of 500 µg total protein from HepG2 lysate or 1000-5362 µg total protein from Caco-2 lysate was diluted in RIPA lysis buffer to 500 µL, added to the antibody-bound Dynabeads, resuspended and incubated with rotation at 4°C overnight to enrich MAO B protein. The next day, the beads were placed on the magnet, lysate supernatant containing unbound molecules was removed and saved in case further analysis was needed. The beads were washed three times with 200 µL wash buffer. The wash solution was saved after each.
wash. To elute bead-bound molecules, the beads were resuspended in 20 µL elution buffer (50 mM glycine, pH 2.8) and rotated at RT for 2 min in a fresh microcentrifuge tube. The eluate was separated from the beads on the magnet, transferred to a fresh microcentrifuge tube, neutralized to pH 7.5 with 1-3 µL of 1 M Tris, pH 8.8 and tested using pH-indicator strips of pH 6.5-10. Finally, the eluate was diluted with 4× Laemmli buffer (Laemmli buffer final concentration 1×) and analyzed for MAO B protein expression by Western blotting as described in Section 2.6 with mouse monoclonal anti-MAO B antibody (Santa Cruz Biotechnology, Dallas, TX).

2.5 ELISA Anti-Human IL-8

For the differentiated Caco-2 cell model, Caco-2 cells were seeded at 2.5×10^4 cells/0.1 mL/insert in 6.5 mm Ø inserts in 24-well plates and cultured in DMEM containing 10% FBS for 17 days (Figure 2.2). Fresh media was changed every other day. On Day 13, cells were brought to quiescence in DMEM containing 2.5% FBS for 24 h before the experiment. Treatments were performed at the end of Day 14 (Figure 2.2). At the time of treatment, culture media were renewed, vehicle control (sterile type 1 H2O) or stimulants (1 µg/mL LPS alone, 20 µM deprenyl alone, 20 µM Compound B alone, 1 µg/mL LPS and 20 µM deprenyl, 1 µg/mL LPS and 20 µM Compound B) were diluted in fresh DMEM with 2.5% FBS to the appropriate concentrations, added to both the apical (100 µL) and the basal (600 µL) media compartments, and incubated for 12 hours. Cells co-treated with LPS and deprenyl or Compound B were pre-treated with 20 µM deprenyl alone or 20 µM Compound B alone for 1 h prior to the addition of LPS. For the undifferentiated Caco-2 cell model, Caco-2 cells were seeded at 2.5×10^4 cells/0.1 mL/well in 96-well microplates and cultured in DMEM containing 10% FBS for 48 h (Figure 2.2). Cells were cultured in DMEM containing 2.5% FBS for another 24 h to reach quiescence prior to
stimulation. Treatments were performed at the end of Day 4 (Figure 2.2). Cells were treated with vehicle control or stimulants as mentioned above (100 µL per well). All treatments were performed in fresh DMEM with 2.5% FBS in biological triplicates. Treatments were completed on Day 15 for differentiated Caco-2 cells and on Day 5 for undifferentiated Caco-2 cells, respectively (Figure 2.2).

Upon completion of treatments, culture media were collected separately while kept on ice, each spiked with 10% BSA solution (final concentration of 1% w/v), and an aliquot of 50 µL from each sample was added per well in the Human IL-8 assay plate (MSD, Rockville, MD) pre-coated with anti-hIL-8 capture antibody and incubated at RT for 2 h with shaking. For the differentiated Caco-2 model, both the apical and the basal media from the same well were collected and added in the assay plate separately. A serial dilution of standards made with hIL-8 calibrator (MSD, Rockville, MD) in DMEM containing 2.5% FBS and 1% BSA was also added to the plate in technical duplicates. After incubation with sample and calibrator solutions, the plate was washed 3 times with 150 µL of PBS containing 0.05% Tween-20 per well, and incubated with 25 µL of anti-hIL-8 detection antibody solution (1 µg/mL) per well at RT for 2 h with shaking. The plate was washed three times, then 150 µL of 2× Read Buffer T (MSD, Rockville, MD) was added per well, and the plate was immediately read with SECTOR S600 instrument (MSD, Rockville, MD). The data were generated by Discovery Workbench software version 4.0 (MSD, Rockville, MD) and analyzed using GraphPad Prism 7 for Mac OS X, Version 7.0c.
2.6 Western Blotting

To examine MAO B protein expression, HepG2 and Caco-2 cells were grown in T75 TC-treated flasks until 90% confluent, in addition, Caco-2 cells were grown in 25 mm ø inserts until 7-days and 14-days differentiated. Whole cell lysates were harvested in RIPA lysis buffer on Day 5 for HepG2 and undifferentiated Caco-2 cells, and on Day 7 and Day 14 for differentiated Caco-2 cells, respectively (Figure 2.2). The procedures were described in Section 2.2 and Section 2.3. 75 μg of total protein from each lysate were diluted with type 1 H₂O and 4× Laemmli buffer (final concentration 1×), and boiled for 5 min immediately before SDS-PAGE and Western blotting.

To study NF-κB and MAPK signaling pathways in undifferentiated Caco-2 cells, cells were seeded at 4-5×10⁵ cells/well/2 mL in 6-well tissue-culture plates and cultured in DMEM containing 10% FBS for 72 h. Cells were washed once with serum-free DMEM and serum starved in serum-free DMEM overnight. The next day, cells were treated with 1 μg/mL LPS for 5 min, 15 min, 30 min, 60 min, 120 min, 180 min and 240 min. LPS was pre-diluted to 10 μg/mL in sterile type 1 H₂O, and 220 μL was directly spiked into the existing media of cell cultures (2 mL/well) without any fresh media change; 220 μL sterile type 1 H₂O was added to the untreated control. To study target pathways in differentiated Caco-2 cells, cells were seeded at 2.5×10⁴ cells/0.1 mL/insert in 6.5 mm ø inserts in 24-well plates and cultured in DMEM containing 10% FBS for 17 days (Figure 2.2). Fresh media was changed every other day. At the end of Day 13, cells were washed once with serum-free DMEM and serum starved in serum-free DMEM overnight. On Day 14, cells were treated with 1 μg/mL LPS for 15 min, 30 min, 60 min, 90 min, 120 min and 240 min. LPS was pre-diluted to 10 μg/mL in sterile type 1 H₂O, 11 μL and 67 μL were directly spiked into the existing apical (100 μL) and basal (600 μL) media of cell
cultures, respectively, without any fresh media change; 11 µL and 67 µL sterile type 1 H2O was added to the untreated control. In some wells, cells were treated with MEK1 inhibitor PD98059 (50 µM) for 2 h. To study target pathways in Raw264.7 cells, cells were seeded at 1.5×10^6 cells/well/2 mL in 6-well tissue culture plates and cultured in DMEM containing 10% FBS for 24 h. The next day, cells were treated with 100 ng/mL LPS for 5 min, 15 min, 30 min, 40 min and 60 min or with 500 ng/mL LPS for 10 min, 30 min, 60 min and 120 min. LPS was pre-diluted to 1 µg/mL or 5 µg/mL in sterile type 1 H2O, and 220 µL was directly spiked into the existing cell culture media (2 mL/well) without any fresh media change; 220 µL sterile type 1 H2O was added to the untreated control. Upon completion of LPS stimulation, Caco-2 and Raw264.7 whole cell lysates were collected as described in Section 2.3. 10-40 µL of whole cell lysate from each sample were directly loaded on SDS-PAGE gel for Western blotting. The appropriate volume of lysates used for each experiment was pre-determined by preliminary experiments.

For Western blotting, lysates and PageRuler™ pre-stained protein marker (Thermo Fisher Scientific, Waltham, MA) were loaded on a 10% SDS-PAGE gel and the proteins were separated by electrophoresis at 80 V through the stacking gel and at 160 V through the resolving gel in 1× Tris-glycine SDS running buffer (192 mM glycine, 25 mM Tris, 0.1% w/v SDS). Proteins were wet transferred from the gel onto a PVDF membrane in 1× cold wet transfer buffer (192 mM glycine, 25 mM Tris, 20% Methanol) at 100 V for 1.5 h. The membrane was then blocked in 5 mL of blocking buffer (1:1 blotk™-FL fluorescent blocker and 1× TBS pH 7.6) for 30 min at RT with shaking, and incubated with rabbit polyclonal Picoband™ anti-MAO B [1:1000], mouse monoclonal anti-MAO B [1:500], anti-phospho-NF-κB p65 Ser536 [1:1000], anti-phospho-ERK1/2 [1:1000], anti-phospho-p38 [1:2000] or anti-phospho-JNK [1:5000]
primary antibody overnight at 4°C with shaking. The next day, the membrane was washed three
times with 1× TBS-0.1% Tween-20 (20 mM Tris/HCl pH 7.6, 150 mM NaCl, 0.1% v/v Tween-
20), incubated with the appropriate secondary antibody [1:15000] at RT for 2 h with shaking,
washed again, and visualized with LI-COR Odyssey Imaging System Version 3.0 (LI-COR
Biosciences, Lincoln, NE). Western blots anti-MAO B were re-probed with mouse anti-GAPDH
antibody as loading control; Western blots against phospho-NF-κB p65 and phospho-MAPKs
were stripped if necessary in 1× Restore Fluorescent WB stripping buffer for 20 min at RT with
shaking, and re-probed with anti-total NF-κB p65 [1:1000], anti-total ERK1/2 [1:1000], anti-
total p38 [1:1000], or anti-total JNK [1:1000] primary antibody.

2.7 Immunofluorescence Staining Anti-RelA/NF-κB p65

Caco-2 cells were seeded at 1×10^5 cells/0.5 mL/well on poly-D-lysine-coated glass
coverslips in a 24-well plate and grown in complete DMEM containing 10% FBS, 1% GlutaMax
and 1% penicillin-streptomycin for 24 h. Raw264.7 cells were seeded at 2.5×10^5 cells/0.5
mL/well on uncoated glass coverslips in a 24-well plate and grown in complete DMEM
containing 10% FBS and 1% penicillin-streptomycin for 24 h. The next day, cells were treated
with LPS (1 µg/mL) for 30 min and 4 h, or with IL-1β (10 ng/mL) for 30 min. LPS and IL-1β
were pre-diluted in sterile type 1 H₂O and PBS pH 7.4, respectively, and directly spiked into the
existing media of cell cultures (0.5 mL/well) without fresh media change. Control cells
(including isotype controls and secondary antibody only controls) were left untreated. After
treatments, cells on coverslips were washed with 500 µL PBS_{Ca^{2+}/Mg^{2+}} (1 mM CaCl₂ and 1 mM
MgCl₂ in PBS), fixed in 500 µL 4% paraformaldehyde in PBS at RT for 20 min, washed three
times with 500 µL PBS_{Ca^{2+}/Mg^{2+}}, permeabilized with 500 µL 0.2% Triton-X100 in PBS at RT for
5 min, washed three times with 500 μL PBS\textsuperscript{Ca2+/Mg2+}, transferred from 12-well plates to parafilm-covered petri dishes (150 mm ø), and blocked in 200 μL blocking buffer (5% goat serum, 0.1% BSA and 0.05% Tween-20 in PBS) at RT for 1 h. Cells were then incubated with 100 μL mouse anti-RelA/NF-κB p65 antibody (20 μg/mL), mouse IgG (20 μg/mL) or plain antibody dilution buffer (1% goat serum, 0.1% BSA, 0.05% Tween-20 in PBS) at 4°C in a humidified microenvironment overnight. The next day, cells were washed three times with wash buffer (0.1% BSA and 0.05% Tween-20 in PBS), incubated with 100 μL AlexaFluor 488 goat anti-mouse IgG secondary antibody (10 μg/mL) at RT for 2 h in the dark, washed again, and mounted with ProLong™ Gold Antifade reagent with DAPI upside down on micro slides. Samples remained at RT overnight in the dark to allow mounting media to solidify and afterwards stored at 4°C. Total NF-κB p65 staining was visualized and photographed using Nikon eclipse 80i fluorescence microscope with Nikon DS Qi1Mc Digital Camera and Nikon NIS-Elements software. Images were analyzed by ImageJ software. All images were taken using the same exposure time (200 ms), and adjusted to the same brightness and contrast settings using ImageJ. The experiment was repeated three times and all treatments were performed in biological duplicates.

2.8 Statistical Analysis

Data were analyzed and graphed using Prism software (GraphPad). Data are displayed as means with standard deviations. Data were analyzed by unpaired \textit{t}-test (two independent sample groups) or one-way ANOVA with Tukey multiple comparison post hoc test compared between all groups of means (more than two independent sample groups) assuming homogeneity of variances. When standard deviations between groups were significantly different (p<0.05) as detected by \textit{F}-test or Brown-Forsythe test and/or Bartlett’s test, the data were analyzed by non-
parametric Mann-Whitney test or Kruskal-Wallis test with Dunn’s post hoc test. Differences were recognized as statistically significant when p<0.05.
3 Results

3.1 Downregulation of LPS-Induced IL-8 Secretion by Compound B in Undifferentiated Caco-2 Cells

The effects of LPS and Compound B on IL-8 secretion in undifferentiated Caco-2 cells were measured in the media supernatants of TC-treated plastic-grown Caco-2 cultures treated with 1 µg/mL LPS alone, 20 µM deprenyl alone, 20 µM Compound B alone, 1 µg/mL LPS and 20 µM deprenyl, or 1 µg/mL LPS and 20 µM Compound B for 12 hours. Preliminary studies have determined that 20 µM was the most effective working concentration for Compound B (Tra, 2015). The baseline level of IL-8 protein secretion (7.05 pg/mL) was significantly increased (p<0.0001) by 1 µg/mL LPS alone treatment at 12 hours (78.1 pg/mL); treatment with 20 µM deprenyl alone or 20 µM Compound B alone had no impact on IL-8 baseline secretion (Figure 3.1a). In Figure 3.1b, the effects of LPS with or without drug in LPS-treated and LPS and drug-co-treated cultures are presented as the absolute change in LPS-induced IL-8 secretion beyond the baseline level. At 12 hours, the absolute change in LPS-induced IL-8 secretion (71.05 pg/mL) was significantly reduced (p<0.05) by co-treatment with 20 µM Compound B (48.49 pg/mL), whereas co-treatment with 20 µM deprenyl did not significantly reduce LPS-induced IL-8 secretion (Figure 3.1b).
Figure 3.1 Compound B reduced LPS-induced IL-8 secretion in undifferentiated Caco-2 cells

Caco-2 cells were cultured on 96-well plates in 10% serum for 48 h, conditioned in 2.5% serum for 24 h to reach quiescence, and stimulated with vehicle control (H₂O), 20 µM deprenyl, 20 µM Compound B, 1 µg/mL LPS with or without 20 µM deprenyl or 20 µM Compound B for 12 h. Media supernatants were analyzed for IL-8 secretion by ELISA. n=3. The experiment was repeated 3 times and representative results are shown. (a): one-way ANOVA with Tukey post hoc test, *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001 compared to control. (b): baseline IL-8 secretion was subtracted from all groups and the absolute change in LPS-induced IL-8 secretion is shown. One-way ANOVA with Tukey post hoc test, *p<0.05 compared to LPS.
3.2 MAO B Expression in Undifferentiated Caco-2 Cells

MAO B protein expression, the drug target protein of Compound B, in undifferentiated Caco-2 cells were examined via Western blotting. Previously, MAO B protein expression in undifferentiated Caco-2 cells had been confirmed (Tra, 2015). However, the anti-MAO B antibody used gave false positive result, as informed by Abcam. Therefore, an extensive search for valid antibodies specific against MAO B was conducted (data not shown). Two were selected and used throughout this study. MAO B protein expression in undifferentiated Caco-2 cells was not detected in whole cell lysate via direct Western blotting (Figure 3.2a) nor after enrichment via immunoprecipitation and subsequent Western blotting (Figure 3.2b). In parallel, as a positive control, HepG2 whole cell lysate was immunoprecipitated, MAO B expression was detected in the HepG2 eluate fraction (Figure 3.2b). When more than 5000 µg of total protein from Caco-2 whole cell lysate was incubated with anti-MAO B antibody, no MAO B was recovered in the eluate fraction (Figure 3.2b). In contrast, HepG2 eluate yielded a clear band, when only 500 µg of total protein from HepG2 whole cell lysate was used for anti-MAO B antibody incubation.
Figure 3.2 MAO B protein expression in undifferentiated Caco-2 cells was undetectable
Caco-2 cells were cultured in T75 tissue culture flasks for 5 days and whole cell lysate was
harvested for (a): direct Western blotting anti-MAO B, and (b): IP anti-MAO B. WCL: whole
cell lysate. SN: unbound supernatant. The experiment was repeated 3 times, and representative
results are shown.
3.3 Examination of LPS-mediated Signaling Pathways in Undifferentiated Caco-2 Cells

Previously, it was shown that LPS significantly upregulated IL-8 gene expression in undifferentiated Caco-2 cells, an effect which was significantly reduced by Compound B (Figure 3.1b). Therefore, we examined the common signaling pathways that regulate LPS-induced IL-8 gene expression. Specifically, nuclear translocation of NF-κB, phosphorylation of RelA/NF-κB p65 at Ser536, and phosphorylation of ERK1/2, p38 and JNK MAPKs were examined in undifferentiated Caco-2 cells. In parallel, these pathways were also examined in LPS-treated Raw264.7 cells as a positive control.

3.3.1 LPS Does Not Induce Nuclear Translocation of NF-κB in Undifferentiated Caco-2 Cells

Nuclear translocation is a hallmark of NF-κB activation. NF-κB nuclear translocation was examined via immunofluorescence staining against the RelA/NF-κB p65 subunit. Immunofluorescence staining against NF-κB p65 in Raw264.7 cells (upper panel) and Caco-2 cells (lower panel) compared to their corresponding secondary antibody controls and isotype controls are shown in Figure 3.3a. In Raw264.7 and Caco-2 cells, secondary antibody controls and isotype controls showed no or minimal nonspecific staining (Alexa488) (Figure 3.3a). Immunofluorescence staining against NF-κB p65 in untreated and LPS-treated Raw264.7 cells (upper panel), untreated, LPS- and IL-1β-treated Caco-2 cells (lower panel) are shown in Figure 3.3b. In resting Raw264.7 cells, staining of NF-κB p65 is present in the cytoplasm (Alexa488 staining); when treated with 1 μg/mL LPS, staining of NF-κB p65 is concentrated in the nucleus.
(merges with DAPI staining), indicating that NF-κB has been activated and has translocated from the cytoplasm to the nucleus (Figure 3.3b). Untreated Caco-2 cells and those treated with 1 µg/mL LPS for 30 min or 4 hours showed diffuse cytoplasmic staining of NF-κB p65, indicating that NF-κB nuclear translocation was not induced by 1 µg/mL LPS (Figure 3.3b). In contrast, as a positive control, Caco-2 cells treated with 10 ng/mL IL-1β displayed concentrated staining of NF-κB p65 in the nucleus (merges with DAPI staining) (Figure 3.3b).
Figure 3.3 LPS did not induce NF-κB nuclear translocation in undifferentiated Caco-2 cells

Caco-2 cells were seeded on poly-D-lysine coated glass coverslips, cultured for 24 h in complete DMEM medium, and treated with 1 μg/mL LPS for 30 min or 4 h, or with 10 ng/mL IL-1β for 30 min. Raw264.7 cells were seeded on glass coverslips, cultured for 24 h in complete medium, and treated with 1 μg/mL LPS for 30 min. (a): secondary antibody and isotype controls compared to anti-total RelA/NF-κB p65 staining. (b): anti-total RelA/NF-κB p65 staining in LPS-treated and IL-1β-treated Raw264.7 and Caco-2 cells. The experiments were repeated 3 times, and representative results are shown. NF-κB nuclear translocation was observed in IL-1β-treated Caco-2 cells and LPS-treated Raw264.7 cells.
3.3.2 LPS Does Not Induce Phosphorylation of RelA/NF-κB p65 at Ser536 in Undifferentiated Caco-2 Cells

Activation of NF-κB is accompanied by multiple phosphorylation events on serine, threonine and tyrosine residues in response to external stimuli such as LPS (Christian et al., 2016). One of the most common phosphorylation site is the Ser536 residue on the RelA/NF-κB p65 subunit. Phosphorylation of RelA/NF-κB p65 at Ser536 was examined via Western blotting. In the positive control, treatment with 500 ng/mL LPS in macrophage-like Raw264.7 cells induced phosphorylation of NF-κB p65 at Ser536 just 10 minutes after stimulation (Figure 3.4a). In contrast, stimulation with 1 µg/mL LPS did not induce NF-κB p65 phosphorylation at Ser536 in undifferentiated Caco-2 cells even when the treatment time was extended to 120 min (Figure 3.4b). Total NF-κB p65 was also probed as a loading control. These data indicate that, 1 µg/mL LPS does not induce phosphorylation of NF-κB p65 on Ser536 or NF-κB nuclear translocation in undifferentiated Caco-2 cells, suggesting that the LPS-mediated signaling for IL-8 gene induction is not mediated through these pathways.
Figure 3.4 LPS did not induce phosphorylation of RelA/NF-κB p65 at Ser536 in undifferentiated Caco-2 cells
Caco-2 cells were cultured in 6-well plates for 72 h, serum starved overnight and stimulated with 1 μg/mL LPS for the indicated time points. Raw264.7 cells were cultured in 6-well plates for 24 h following stimulation with 500 ng/mL LPS for the indicated time points. Whole cell lysates were analyzed by Western blotting. **(a)**: LPS induced NF-κB p65 phosphorylation at Ser536 in Raw264.7 cells. **(b)**: LPS did not induce NF-κB p65 phosphorylation at Ser536 in undifferentiated Caco-2 cells. The experiments were repeated at least 3 times and representative results are shown.
3.3.3 LPS Does Not Induce Phosphorylation of ERK1/2, p38 and JNK MAPKs in Undifferentiated Caco-2 Cells

Phosphorylation of ERK1/2, p38 and JNK MAPKs were examined via Western blotting. In the positive control, 100 ng/mL LPS induced phosphorylation of ERK1/2, p38 and JNK by 5 min in macrophage-like Raw 264.7 cells (Figure 3.5a). In contrast, stimulation with 1 µg/mL LPS did not induce phosphorylation of ERK1/2, p38 and JNK in undifferentiated Caco-2 cells even when the treatment time was extended to 240 min (Figure 3.5b). The baseline level of ERK1/2 phosphorylation in untreated cells was high, but cells treated with 50 µM PD98059, an inhibitor specific for MEK1, an upstream kinase of ERK1/2, demonstrated that ERK1/2 signaling was present in untreated cells (Figure 3.5b). Total ERK1/2, p38 and JNK were also probed as loading controls. These data indicate that, 1 µg/mL LPS does not induce the phosphorylation of ERK1/2, p38 and JNK MAPKs in undifferentiated Caco-2 cells, suggesting that the LPS-mediated signaling for IL-8 gene induction is not mediated by these pathways.
Figure 3.5 LPS did not induce phosphorylation of ERK1/2, p38 or JNK MAPKs in undifferentiated Caco-2 cells

Caco-2 cells were cultured in 6-well plates for 72 h, serum starved overnight, and treated with 50 µM PD98059 for 2 hours or 1 µg/mL LPS for the indicated time points. Raw264.7 cells were cultured in 6-well plates for 24 h following stimulation with 100 ng/mL LPS for the indicated time points. Whole cell lysates were analyzed by Western blotting. (a): LPS induced phosphorylation of ERK1/2, p38 and JNK in Raw264.7 cells. (b): LPS stimulation did not induce phosphorylation of ERK1/2, p38 or JNK in undifferentiated Caco-2 cells. The experiments were repeated at least 3 times and representative results are shown.
3.4 MAO B Expression in Differentiated Caco-2 Cells

Caco-2 cells cultured in histotypic conditions (Transwell inserts) mimicking the in vivo environment of the intestine has been shown to dramatically impact cell differentiation (Sun et al., 2008; Yoo et al., 2012; Lea, 2015). Therefore, we examined MAO B protein expression in Transwell-grown Caco-2 cultures on Day 7 and Day 14 via Western blotting (Figure 2.2). MAO B protein expression was induced in 7-days and 14-days Transwell-grown Caco-2 cultures, but not detected in 5-days tissue culture-treated plastic-grown Caco-2 cultures (Figure 3.6a). The level of MAO B expression was time-dependent. Densitometry analysis showed that it increased by 1.1 ± 0.07-fold (Mean ± Range) at 7 days and 11.2 ± 0.08-fold (Mean ± Range) at 14 days relative to GAPDH (Figure 3.6a). The same whole cell lysate samples were blotted using another anti-MAO B antibody, and MAO B protein expression in 14-days differentiated cells was confirmed (Figure 3.6b). Based on preliminary experiments, this antibody had less sensitivity (data not shown). GAPDH was probed as a loading control.
Figure 3.6 MAO B expression is induced in differentiated Caco-2 cells

Caco-2 cells were cultured in 25 mm inserts for 7 days and 14 days after reaching confluency. Whole cell lysates were analyzed by Western blotting. Samples were prepared in biological duplicates. (a): MAO B protein expression was detected in 7-days and 14-days differentiated Caco-2 cells, but not detected in undifferentiated Caco-2 cells. (b): MAO B protein expression was confirmed in 14-days differentiated Caco-2 cells using a less sensitive anti-MAO B antibody. The experiment was repeated three times and representative data are shown.
3.5 Downregulation of LPS-Induced IL-8 Secretion by Compound B in Differentiated Caco-2 Cells

To determine if Compound B downregulates LPS-induced IL-8 secretion in differentiated Caco-2 cells, IL-8 concentrations were analyzed in the apical and basal media of 14-days differentiated Caco-2 cell cultures treated for 12 hours with 1 µg/mL LPS alone, 20 µM deprenyl alone, 20 µM Compound B alone, 1 µg/mL LPS and 20 µM deprenyl, or 1 µg/mL LPS and 20 µM Compound B. In Figure 3.7a, baseline apical IL-8 protein secretion (13.9 pg/mL) was significantly induced (p<0.05) by 1 µg/mL LPS alone treatment at 12 hours (175.36 pg/mL), 20 µM deprenyl alone or 20 µM Compound B alone did not impact IL-8 baseline protein expression. Co-treatment with 20 µM deprenyl or 20 µM Compound B for 12 hours did not have any significant effect on LPS-induced IL-8 secretion to the apical media (Figure 3.7b). Baseline basal IL-8 protein secretion (0.66 pg/mL) was significantly increased (p<0.05) by 1 µg/mL LPS alone at 12 hours (17.28 pg/mL), 20 µM deprenyl alone or 20 µM Compound B alone did not impact baseline IL-8 secretion (Figure 3.7c). In contrast, the absolute change in LPS-induced IL-8 secretion to the basal media (16.61 pg/mL) was significantly reduced (p<0.05) by co-treatment with 20 µM Compound B (11.84 pg/mL) at 12 hours, but there was no significant reduction in IL-8 protein secretion in deprenyl-co-treated cultures (Figure 3.7d).
Figure 3.7 Compound B reduced LPS-induced basal, but not apical, IL-8 secretion in 14-days differentiated Caco-2 cells
Caco-2 cells were cultured in 6.5 mm inserts in 10% serum for 14 days after reaching confluency to induce differentiation, conditioned in 2.5% serum for 24 h to reach quiescence, and stimulated with vehicle control (H₂O), 20 µM deprenyl, 20 µM Compound B, 1 µg/mL LPS with or without 20 µM deprenyl or 20 µM Compound B for 12 h. Apical and basal culture media were analyzed separately for IL-8 secretion by ELISA. n=4. The experiments were repeated 3 times and representative results are shown. (a-b): Compound B did not reduce LPS-induced apical IL-8 secretion in differentiated Caco-2 cells. (c-d): Compound B effectively reduced LPS-induced basal IL-8 secretion in differentiated Caco-2 cells, but deprenyl did not. (a) and (c): Kruskal-Wallis test with Dunn’s post hoc test, *p<0.05 compared to control. (b) and (d): baseline IL-8 secretion is subtracted from all groups and the absolute level of LPS-induced IL-8 secretion is shown. (b): one-way ANOVA with Tukey post hoc test. (d): Kruskal-Wallis test with Dunn’s post hoc test, *p<0.05 compared to LPS.

The % reduction of LPS-induced IL-8 secretion by Compound B was calculated for undifferentiated and 14-days differentiated Caco-2 cultures. The baseline level of IL-8 secretion was subtracted from the LPS-induced IL-8 secretion (pg/ml) and that reduced by Compound B...
(pg/ml) to obtain the absolute change in LPS-induced IL-8 secretion (Equation 3.1). The mean % reductions of LPS-induced IL-8 secretion by Compound B in undifferentiated and 14-days differentiated Caco-2 cell cultures from three independent experiments with standard deviations are presented in Table 3.1. Although MAO B protein expression was present in differentiated Caco-2 cells (Figure 3.6) but absent in undifferentiated Caco-2 cells (Figure 3.2 and Figure 3.6), the mean % reductions of LPS-induced IL-8 secretion by Compound B between the two cell culture conditions are not significantly different (p=0.675) (Table 3.1). These data suggest the IL-8 suppressing effect of Compound B appear to be independent of MAO B protein expression.

**Equation 3.1 % reduction of LPS-induced IL-8 secretion reduced by Compound B**

\[
\text{% reduction} = \left( 1 - \frac{LPS\text{-induced IL-8 secretion reduced by Compound B (pg/ml)}}{LPS\text{-induced IL-8 secretion (pg/ml)}} \right) \times 100
\]

**Table 3.1 Comparison of mean % reductions of LPS-induced IL-8 secretion by Compound B between undifferentiated and 14-days differentiated Caco-2 cultures**

The % reductions of LPS-induced IL-8 secretion in undifferentiated and 14-days differentiated Caco-2 cell cultures are calculated using Equation 3.1. Data are presented as mean ± SD. NS: not significant.

<table>
<thead>
<tr>
<th>Caco-2 culture condition</th>
<th>Undifferentiated</th>
<th>14-Days Differentiated</th>
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<td>Media compartment</td>
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<td></td>
<td>Basal</td>
<td>Apical</td>
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<tr>
<td>Mean % reduction of LPS-induced IL-8 secretion by Compound B (± SD)</td>
<td>35.27 ± 3.86%</td>
<td>33.61 ± 5.07%</td>
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<td>MAO B protein expression (Figure 3.6)</td>
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</table>
3.6 LPS Does Not Induce Phosphorylation of RelA/NF-κB p65 at Ser536, ERK1/2, p38 and JNK MAPKs in Differentiated Caco-2 Cells

To determine if LPS induced IL-8 secretion in 14-days differentiated Caco-2 cells via activation of the common signaling pathways regulating TLR-mediated IL-8 gene expression, phosphorylation of RelA/NF-κB at Ser536, and phosphorylation of ERK1/2, p38 and JNK MAPKs were examined via Western blotting. Treatment with 1 µg/mL LPS did not induce phosphorylation of NF-κB p65 at Ser536 (Figure 3.8a), ERK1/2 (Figure 3.8b), p38 (Figure 3.8c) and JNK (Figure 3.8d) MAPKs in 14-days differentiated Caco-2 cells at the indicated time points. As a negative control, cells were treated with 50 µM PD98059, an inhibitor specific for MEK1, an upstream kinase of ERK1/2. Total NF-κB p65, ERK1/2, p38 and JNK proteins were probed as loading control. In conclusion, 1 µg/mL LPS does not induce the activities of NF-κB p65, ERK1/2, p38 and JNK MAPKs via phosphorylation in 14-days differentiated Caco-2 cells, suggesting that the LPS-mediated signaling for IL-8 gene induction was not mediated by these pathways. Therefore, they are not the targets for the suppressing effect of Compound B on LPS-induced IL-8 gene and protein expression.
Figure 3.8 LPS did not induce phosphorylation of RelA/NF-κB p65 at Ser536, ERK1/2, p38 or JNK in 14-days differentiated Caco-2 cells

Caco-2 cells were cultured in 6.5 mm inserts for 14 days after reaching confluency, serum starved overnight and treated with 1 µg/mL LPS on Day 18. Whole cell lysates were analyzed by Western blotting. LPS did not induce (a): RelA/NF-κB p65 at Ser536, (b): ERK1/2, (c): p38, or (d): JNK in 14-days differentiated Caco-2 cells. The experiments were repeated 3 times and representative results are shown. Samples in (b) and (c) were prepared in biological duplicates.
4 Discussion

4.1 Novel MAO B Inhibitor Downregulates LPS-induced Cytokine Expression

MAO B inhibition has been shown to downregulate cytokine expression in various disease models. In a rat model of renal ischemia-reperfusion injury, pargyline treatment decreased the gene expression of pro-inflammatory cytokines IL-1β, TNF-α and profibrotic cytokine TGF-β (Chaaya et al., 2010). In a rat model of chronic periodontal disease, phenelzine treatment reduced LPS-induced TNF-α expression (Ekuni et al., 2009). Consistent with these findings, we showed that novel MAO B inhibitors inhibited MAO B in vitro and downregulated LPS-induced gene expression of pro-inflammatory cytokines IL-6, IL-8 and TNF-α in intestinal epithelial and microvascular endothelial cell models (Tra, 2015). Such inhibitory effects may potentially be due to the reduction of H₂O₂ levels as the result of inhibition of MAO B enzyme activity, since high levels of H₂O₂ have been suggested to be involved in the process of tissue injury and induction of cytokine expression (Kunduzova et al., 2002; Schieber and Chandel, 2014).

4.2 MAO B Protein Expression in Undifferentiated and Differentiated Caco-2 Cultures

Prolonged culture in Transwell inserts induces spontaneous differentiation in Caco-2 cells (Sun et al., 2008; Yoo et al., 2012; Lea, 2015). During differentiation, they undergo noticeable changes in shape, structure, protein composition and barrier function to become in vivo-like, resembling normal intestinal epithelial absorptive cells. Differentiated Caco-2 cells form tight junctions between adjacent cells, which increases the barrier integrity of the epithelium. They
also develop apical and basal membranes with distinct morphologies, enzyme and transporter expression profiles and biochemical functions, such as microvilli on the apical membrane (Sun et al., 2008; Yoo et al., 2012; Lea, 2015). Previously, it was shown that MAO B protein expression was upregulated during Caco-2 cell differentiation, especially after 14 days of post-confluent culturing (Wong et al., 2003). In undifferentiated Caco-2, the MAO B promoter region is heavily methylated, and MAO B gene expression is repressed. During Caco-2 cell differentiation, DNA demethylation of the MAO B promoter exposes it to transcription factors that activate MAO B gene expression (Wong et al., 2003). Our results are consistent with this finding. Our study found MAO B protein expression was detectable in Caco-2 cells cultured in Transwell inserts (Figure 3.6), but not in Caco-2 cells cultured on tissue culture-treated plastic as examined by IP and Western blotting (Figure 3.2 and Figure 3.6). Interestingly, the absence of MAO B did not affect the relative anti-inflammatory property of Compound B. In fact, Compound B demonstrated equal efficacy in both undifferentiated and differentiated Caco-2 cultures, as measured by the % reduction of LPS-induced IL-8 secretion (Table 3.1). These results support a hypothesis that downregulation of LPS-induced IL-8 secretion by Compound B is independent of MAO B protein presence in Caco-2 cells. It is unlikely that the downregulating effect arises from inhibition of MAO A, because Compound B has been shown to selectively inhibit MAO B and not MAO A (Figure 1.3).

4.3 LPS Regulation of IL-8 Expression in Caco-2 Cultures

Even though no MAO B protein expression was detected, the fact that Compound B still reduced the level of LPS-induced IL-8 secretion in undifferentiated Caco-2 cells led to our investigation in the common LPS-activated intracellular signaling pathways that mediate IL-8
gene expression, which could possibly be interfered by Compound B. Nuclear translocation and phosphorylation of NF-κB and MAPKs are the common pathways that control LPS-activated IL-8 gene expression (Hoffmann et al., 2002; Roux and Blenis, 2004; Chen and Greene, 2004; Viatour et al., 2005; Vermeulen et al., 2006; Christian et al., 2016). Unexpectedly, we demonstrated that LPS alone did not induce NF-κB nuclear translocation, phosphorylation of NF-κB p65 subunit at Ser536, or phosphorylation of MAPKs in undifferentiated (Figure 3.3, Figure 3.4 and Figure 3.5) or 14-days differentiated (Figure 3.8) Caco-2 cell cultures. Murine macrophage-like Raw264.7 cells treated with LPS exhibited NF-κB nuclear translocation and phosphorylation of NF-κB and MAPKs. These positive results confirm the virulence of LPS and validate the experimental design. Cell surface receptor TLR4 specifically recognizes LPS and is responsible for LPS responsiveness of cells (Takeda and Akira, 2005; O'neill et al., 2013; Vaure and Liu, 2014). Current literature provides contradicting evidences regarding TLR4 gene and protein expression in the Caco-2 cell line. Many groups reported that Caco-2 cells did not express TLR4 mRNA or protein, or lacked other essential components of the TLR4 complex, such as MyD88 and MD-2, despite positive TLR4 and CD14 protein expression, thus remained unresponsive to LPS stimulation in terms of IL-8 secretion, activation of NF-κB, and phosphorylation of MAPKs (Abreu et al., 2001; Funda et al., 2001; Naik et al., 2001; Böcker et al., 2003; Suzuki et al., 2003; Tyrer et al., 2006; Lenoir et al., 2008; Hsu et al., 2011; Kuo et al., 2015). Techniques used to examine the expression of TLR4 complex components in the above studies include RT-PCR, Western blot analysis, immunocytochemistry and flow cytometry analysis. Transient co-transfection of TLR4 and MD2 restored LPS responsiveness in Caco-2 cells as measured by increased NF-κB and IL-8 reporter gene activities (Abreu et al., 2001). These findings may explain the negative signaling results from Caco-2 cells in our study. Indeed,
it is believed that normal intestinal epithelium expresses low levels of TLR4 as a mechanism to develop tolerance towards luminal LPS found on commensal Gram-negative bacteria, as it is constantly exposed to the gut flora in healthy condition (Abreu et al., 2001; Naik et al., 2001; Suzuki et al., 2003; Vaure and Liu, 2014).

In contrast, some studies showed that TLR4 mRNA and protein were detected in Caco-2 cells (Furrie et al., 2005; Cario et al., 2000; Abreu, 2010; Tyrer et al., 2006; Hsu et al., 2011). In our study, LPS consistently and significantly increased the levels of IL-8 gene and protein expression compared to the unstimulated and drug alone-treated controls in both undifferentiated (Figure 3.1) and 14-days differentiated Caco-2 cells (Figure 3.7). Caco-2 cells utilized in these experiments were responsive to LPS in terms of IL-8 gene and protein expression, but is driven by alternative mechanisms independent of the NF-κB and MAPKs signaling pathways.
4.4 Conclusions

This study is a significant attempt to understand the effect of MAO B inhibition and its mechanism of action in an *in vitro* model of mucosal inflammation. The effects of LPS and the novel MAO B inhibitor Compound B on IL-8 protein expression in Caco-2 cells were studied by ELISA. MAO B protein expression in Caco-2 cells was examined by Western blotting and immunoprecipitation. Western blotting and immunofluorescence staining were applied to study the signaling pathways regulating IL-8 gene and protein expression in Caco-2 cells stimulated with LPS. Our study demonstrates that, in Caco-2 cells:

- Compound B downregulates LPS-induced IL-8 protein expression;
- MAO B protein expression does not impact the efficacy of Compound B, leading to a hypothesis that downregulation of IL-8 gene and protein expression by Compound B appears to be independent of MAO B protein expression and mediated by alternative mechanisms;
- LPS-induced IL-8 gene and protein expression is not mediated by the NF-κB and MAPKs signaling pathways, thus, downregulation of LPS-induced IL-8 gene and protein expression by Compound B does not affect these pathways;
- To identify potential LPS-mediated cytokine signaling pathways that are being downregulated by Compound B, a more global approach is required to identify protein binding partners of Compound B.
4.5 Future Direction

Reduction of LPS-mediated cytokine expression by Compound B may be due to drug-protein interactions between Compound B and proteins other than MAO B. Understanding this potential off-target effect poses a challenge to us.

We will first confirm that cytokine downregulation by Compound B is independent of the presence or absence of MAO B protein. We will utilize the human liver epithelial cell line HepG2 (known MAO B expressing cell line) and knock-down MAO B expression in HepG2 cells using RNAi technology. If cytokine downregulation by Compound B is indeed independent of MAO B protein expression, we can then move forward with identifying other Compound B protein binding partners using SILAC analysis.

SILAC analysis is a state of the art technique for studying the quantitative proteomics of cell cultures. It combines isotope-labeling of cellular proteins and LC-MS to detect protein-drug interactions in the cell (Ong et al., 2009). To identify proteins that bind to a small-molecule drug, arginine and lysine labeled with light (untreated control) or heavy (drug-treated) isotopes are added to cell culture media and incorporated into cellular proteins by cells during protein synthesis. Cells are stimulated with or without the drug, mechanically lysed, fractionated by size-exclusion chromatography, and analyzed by MS and software for protein identification (Kristensen and Foster, 2014). Specific bindings between drug and proteins are defined by co-elution of proteins and the drug. Proteins that bind both directly and indirectly to the drug can be detected. Isotope-labeling of proteins allows detection of co-elution without time-consuming and labor-intensive protein tagging and purification (Kristensen et al., 2012). The SILAC approach of studying drug effects has been validated by various studies. It revealed the mechanism of selective killing of cancer cells by a small-molecule drug, piperlongumine, and the mechanism of
intestinal organoids growth inhibition by an HDAC inhibitor, CI994, through identification of their protein binding partners (Raj et al., 2011; Gonneaud et al., 2016). Therefore, SILAC is a promising method to identify protein binding partners of Compound B and investigate its mechanism of action in Caco-2 cells and other valid cell models.
Bibliography


lipopolysaccharide is correlated with Toll-like receptor 4 but not Toll-like receptor 2 or CD14 expression. *International Journal of Colorectal Disease, 18*(1), 25-32.


