THYMOQUINONE IS A NOVEL LIGAND WHICH ACTIVATES NEU4 SIALIDASE TO PROMOTE A PRO-INFLAMMATORY RESPONSE

by

Trisha M. Finlay

A thesis submitted to the Department of Microbiology and Immunology
In conformity with the requirements for
the degree of Master’s of Science

Queen’s University
Kingston, Ontario, Canada
(April, 2009)

Copyright ©Trisha M. Finlay, 2009
Abstract

Thymoquinone (TQ), a volatile oil component of black seed oil (derived from *Nigella sativa*), has been shown to have various biological effects including disease treatment and prevention. TQ is believed to share similar properties to the benzoquinones already in use as therapeutic drugs. Based on previous reports on the anti-inflammatory properties of black seed oil and TQ, it was originally hypothesized that TQ would inhibit lipopolysaccharide (LPS)-induced cellular sialidase activity in an anti-inflammatory manner. Sialidase activity was tested on live mouse bone marrow derived primary macrophage cells, BMC-2 macrophage cells, human embryonic kidney epithelial (HEK293) cells and human fibroblast cells using an assay that measures the cleavage of the sialidase specific fluorescent substrate 2’-(4-methylumbelliferyl)-α-DN-acetylneuraminic acid (4-MUNANA). The cleavage of 4-MUNANA causes the release of free 4-methylumbelliferone, which fluoresces at 450nm (blue) after excitation at 365nm. Unexpectedly, TQ induced sialidase activation in all three cell lines and wild type primary macrophage cells. TQ was unable to induce sialidase activity in primary macrophage cells isolated from Neu4 knockout mice suggesting that the TQ activates Neu4 sialidase enzyme. TQ-induced sialidase activity in these live cells was found to occur through intermediate GPCR-associated guanine nucleotide Gαi subunit and matrix metalloproteinase 9 (MMP9) by using specific inhibitors. In addition, TQ was found to induce sialidase activity in Toll-like receptor-deficient HEK293 cells. These latter data suggested that TQ may be activating GPCR Gαi and MMP9 signaling associated with Neu4 sialidase independent of TLRs. It is proposed that TQ-induced sialidase activity may activate Toll-like receptors in macrophage cells and the subsequent production of pro-inflammatory cytokines in the absence of LPS. Immunocytochemical staining of BMC-2 cells shows that TQ induced NFκB activation. NFκB activation was confirmed with electrophoretic mobility shift assay (EMSA) and western immunoblotting techniques. Cytokine arrays were used to test the pro-inflammatory
cytokine response induced in mice by 5 hour treatment of TQ, compared to LPS. Mice treated with TQ exhibited an increase in IL-1β, IL-6 and TNF-α production, similar to LPS treatment. Taken together, the findings in these studies suggest that TQ is a novel ligand for Neu4 sialidase activation which consequently induces pro-inflammatory cytokine responses.
Acknowledgements

Queen’s University has been my home for 6 years. Throughout the journey here, I have been blessed with wonderful friends and mentors. I could not have completed this work without the amazing support of my family and friends along the way.

I would like to thank Dr. Szewczuk for taking that first chance on me to undertake a 4th year project in his lab and for putting his faith in me to continue as a Masters student. His generous support and helpful guidance have been invaluable. He encourages all of us to get out there and share out work in the scientific community. Mostly he is a constant motivation to reach for more successes in the future.

To my supervisory and examination committee members, Dr Gee and Dr. Elliott, your support and guidance have also been indispensable. Dr. Gee has kept me on the right path and encourages me to increase the depth in both my research and writing. Dr. Elliott has had unfailing words of advice and helpful suggestions due to his extensive knowledge in cancer biology and matrix metalloproteinases. Thank you to Dr. Raptis for stepping into the role of Head delegate with only a short notice. Thank you to Dr. Kan for chairing for my examination. Dr. Basta and Dr. Pshezhetsky have also supported my research and journey as a graduate student. The Microbiology and Immunology department must be thanked for their support and assistance with my project.

To Ray and Preethi: I couldn’t imagine doing this without you. Your love and support in and out of the lab has been the most phenomenal part of the last 3 years. I can never thank you enough for all that you have done to support me. Ray thank you for all of your generous help with my writing skills and being the best mentor anyone could ask for. Preethi: thank you for always being there to lend a helping hand and listen to me when I needed a friend. I will never forget our wonderful
adventure to Texas (Also, thank you to Arshia for staying inside while we were traveling). Thanks to Shahrul for helping me throughout my undergraduate project and introducing me to Thymoquinone. Susan you made my life so much easier while I was running around trying to finish my thesis. Thank you for taking over the lab and doing such a wonderful job helping everyone. The Immunogoblins, Christina, Ila, Sarah and Tara, have provided me with priceless support and precious friendships that will last a lifetime. Thanks to Charmaine for her help as my volunteer. Teaching her was a delightful experience.

Finally, thank you to my family and friends back home. My friends have been my relief from the pressures of school and are always around to pick me up. Thank you to Dylan; you are the song that fills my heart. To my brother for never failing to bring a smile to my face no matter what my situation is. My parents are a priceless gift. They are the most giving, caring people I know. You are my motivation for greatness, my passion for life and my goal for future happiness. Mom: thank you for Tuesdays.

This thesis is dedicated to my parents and Ray and Preethi without which I would never have been able to complete this endeavor.
Table of Contents

Abstract ...................................................................................................................................................... ii
Acknowledgements ........................................................................................................................................ iv
Table of Contents ......................................................................................................................................... vi
List of Figures ................................................................................................................................................ ix
List of Abbreviations ...................................................................................................................................... xii
Chapter 1 Introduction ................................................................................................................................. 1
  1.1 *Nigella sativa* and Thymoquinone .................................................................................................................. 1
  1.2 Inflammation .................................................................................................................................................... 2
  1.3 Toll-Like Receptors .......................................................................................................................................... 3
  1.4 Sialidase .......................................................................................................................................................... 3
  1.5 G-Protein Coupled Receptors .......................................................................................................................... 4
  1.6 Matrix Metalloproteinase ............................................................................................................................... 5
  1.7 Rationale, Objectives and Hypothesis ............................................................................................................. 5
Chapter 2 Literature Review .......................................................................................................................... 7
  2.1 The Medicinal Properties of TQ ...................................................................................................................... 7
    2.1.1 TQ & Cancer ............................................................................................................................................. 7
    2.1.2 TQ & Diabetes ......................................................................................................................................... 10
    2.1.3 TQ & Inflammation ............................................................................................................................... 11
  2.2 Sialidase and Inflammation ............................................................................................................................ 13
    2.2.1 Sialidase and TLR4 ............................................................................................................................... 13
    2.2.2 Neu4 Sialidase .......................................................................................................................................... 14
    2.2.3 MMP, GPCR and Sialidase activity ......................................................................................................... 15
Chapter 3 Materials and Methods ................................................................................................................ 17
  Cell Lines ......................................................................................................................................................... 17
  Mouse Models .................................................................................................................................................. 18
  Primary mouse bone marrow derived macrophage cells .................................................................................... 18
  Ligands ............................................................................................................................................................. 19
  Inhibitors .......................................................................................................................................................... 19
  Antibodies ........................................................................................................................................................ 22
  Live Cell Sialidase Activity - Sialidase Assay ..................................................................................................... 23
  Sialidase Standardization .................................................................................................................................. 24
Immunocytochemistry of anti-Neu and anti-MMP9 antibodies ........................................... 24
Immunocytochemistry of NFκB ......................................................................................... 25
TLR4 co-localization with MyD88 .................................................................................... 25
Cell Lysates ...................................................................................................................... 26
Nuclear extracts ............................................................................................................... 26
Bradford assay ................................................................................................................ 27
Electrophoretic mobility shift assay (EMSA) .................................................................... 28
Western Blot Analysis ...................................................................................................... 28
mRNA extracts and polymerase chain reaction (PCR) ...................................................... 29
Cytokine array profiling ................................................................................................... 30
Cell Viability .................................................................................................................. 31
Cell Morphology ............................................................................................................ 31
Chapter 4 Results ........................................................................................................... 33
4.1 TQ activates sialidase in macrophage cells ................................................................. 33
  4.1.1 Quantification of sialidase activity ........................................................................ 33
  4.1.2 Black seed oil and component para-Cymene inhibit ligand-induced sialidase activation .......................................................................................................................... 33
  4.1.3 Black seed oil component Thymoquinone induces sialidase activity .................. 36
4.2 TQ preferentially activates Neu4 ............................................................................. 42
  4.2.1 Anti-Neu antibodies were able to bind to non-permeabilized and permeabilized mouse macrophage cells ................................................................. 42
  4.2.2 Anti-Neu4 antibody completely inhibited TQ-induced sialidase activation in mouse BMC-2 macrophage cells and human HEK-TLR4 cells .................. 47
  4.2.3 Sialidase activity was measured in primary bone marrow derived macrophages isolated from Neu4 knock-out mice to confirm Neu4 activation by TQ treatment ......................... 54
4.3 TQ induces a pro-inflammatory response through sialidase Neu4 ............................ 59
  4.3.1 TQ treatment of macrophage cells stimulates NFκB activation and nuclear localization ................................................................. 59
  4.3.2 Neuraminidase inhibitor Tamiflu inhibits TQ-induced NFκB activation ............... 64
  4.3.3 TQ treatment in mice up regulates pro-inflammatory cytokine production in vivo .... 69
  4.3.4 TQ-induced expression of pro-inflammatory cytokines is dependent on Neu4 sialidase ............................................................................................................................. 72
4.3.5 TQ up regulates key pro-inflammatory cytokine genes ............................................................ 72
4.4 TQ activates Neu4 sialidase through GPCR Ga, subunit and MMP9 ........................................ 82
  4.4.1 MMP and GPCR Ga, subunit inhibitors are able to diminish TQ-induced sialidase activity ........................................................................................................................................... 82
  4.4.2 MMP9 is involved in TQ-induced sialidase activity ................................................................ 85
  4.4.3 Both GPCR Ga, subunit and MMP9 are involved in TQ-induced NFκB activation ... 92
4.5 TQ trans-activates TLR4 through Neu4 ...................................................................................... 97
  4.5.1 TQ is not a TLR4 ligand .......................................................................................................... 97
  4.5.2 TQ activates TLR4 in wild type and NeuIn mouse primary macrophage cells ........ 97
  4.5.3 TQ is unable to activate TLR4 in Neu4 knock out macrophage cells ................... 98
  4.5.4 GPCR Ga, subunit and MMP9 are important for TQ-induced TLR4 activation ...... 103
4.6 Cell morphology following treatment with TQ ........................................................................ 106
  4.6.1 TQ treatment does not affect cell viability .......................................................... 106
  4.6.2 Morphology of cells affected by TQ treatment .................................................................. 109
Chapter 5 Discussion .................................................................................................................... 112
References ........................................................................................................................................ 128
Appendix A Supplemental Data .................................................................................................... 133
List of Figures

Figure 1. Structural diagrams of inhibitors.................................................................20
Figure 2. Standardization of enzyme activity in live cells sialidase assay performed with C.
perfringens derived neuraminidase..............................................................................34
Figure 3. Dose dependent TQ-induced sialidase activity in BMC-2 macrophage cells ..........37
Figure 4. TQ solvents do not induce sialidase activity in BMC-2 cells and TQ does not exhibit
sialidase activity in the absence of cells........................................................................40
Figure 5. Identification of membrane-associated Neu sialidase in BMC-2 macrophage cells ......43
Figure 6. Identification of membrane-associated Neu sialidase in wild type mouse primary
macrophage cells............................................................................................................45
Figure 7. Neu4 sialidase is induced by TQ treatment in live BMC-2 cells........................48
Figure 8. Identification of membrane-associated Neu sialidase induced by TQ treatment in HEK-
TLR4 cells.......................................................................................................................50
Figure 9. Identification of Neu sialidase induced by TQ treatment of BMC-2 cells with Anti-Neu
antibodies and DANA.......................................................................................................52
Figure 10. TQ-induced sialidase activity in wild type mouse primary macrophage cells and Neu1
deficient human fibroblast cells......................................................................................55
Figure 11. TQ treatment of live bone marrow derived primary macrophage cells isolated from
Neu4 knockout mice does not induce sialidase activity..................................................57
Figure 12. TQ-induced NFκB activation in BMC-2 macrophage cells..............................60
Figure 13. TQ-induced NFκB activation in HEK-TLR4 cells...........................................62
Figure 14. Nuclear localization and DNA binding of TQ-induced NFκB p65 activation in......65
Figure 15. Tamiflu blocks TQ-induced NFκB activation in BMC-2 macrophage cells ..........67
Figure 16. Cytokine array membranes used for the analysis of TQ-induced cytokine production in
serum from wild type, Neu1 deficient (NeuIn), and Neu4 knock-out (Neu4KO) mice ......70
Figure 17. Analysis of TQ-induced pro-inflammatory cytokine production in serum from wild
type, Neu1 deficient and Neu4 knockout mice...............................................................73
Figure 18. Analysis of TQ-induced pro-inflammatory chemokine production in serum from wild
type, Neu1 deficient and Neu4 knockout mice...............................................................75
Figure 19. Analysis of TQ-induced production of other cytokines in serum from wild type, Neu1
deficient and Neu4 knockout mice................................................................................77
Figure 20. TQ-induced increase in expression of mRNA of pro-inflammatory cytokines: IL-6, TNF-α and MIP-1β ................................................................. 80
Figure 21. TQ-induced sialidase activity is inhibited with sialidase, GPCR Gαi subunit and MMP inhibitors in BMC-2 macrophage cells ................................................................. 83
Figure 22. TQ-induced sialidase activity is inhibited by sialidase, GPCR Gαi subunit and MMP inhibitors in primary macrophage cells from wild type mice ............................................ 86
Figure 23. TQ-induced sialidase activity in BMC-2 cells is inhibited by MMP inhibitor piperazine .......................................................................................................................... 88
Figure 24. TQ-induced sialidase activity in BMC-2 cells is inhibited by the MMP inhibitor galardin and MMP9 neutralizing antibody ........................................................................ 90
Figure 25. Detection of membrane associated MMP9 in BMC-2 macrophage cells and wild type mouse primary macrophage cells................................................................. 93
Figure 26. TQ-induced NFκB activation is reduced by sialidase, GPCR Gαi subunit, and MMP inhibitors .................................................................................................................. 95
Figure 27. TQ-induced MyD88 co-localization with TLR4 in primary macrophage cells isolated from wild type and Neu1 deficient mice ...................................................................... 99
Figure 28. Lack of TQ-induced MyD88 co-localization with TLR4 in primary macrophages cells isolated from Neu4 knockout mice ........................................................................... 101
Figure 29. Tamiflu, Galardin and Pertussis Toxin inhibit TQ-induced MyD88 co-localization with TLR4 in primary macrophage cells isolated from wild type mice......................... 104
Figure 30. Cell Viability in HEK-TLR4 cells treated with TQ and sialidase inhibitor Tamiflu . 107
Figure 31. Cell morphology study of BMC-2 cells treated with TQ and Tamiflu ......................... 110
Figure 32. Experimental Model 1: TQ-induced sialidase activity through GPCR Gαi-MMP9-Neu4 .......................................................................................................................... 117
Figure 33. Experimental Model 2: TQ induces sialidase activity through MMP9-GPCR Gαi-Neu4 .......................................................................................................................... 119
Figure 34. Experimental Model 3: TQ-induced activation of Neu4 leads to subsequent activation of TLR4.................................................................................................................... 123
Figure 35. Ligand-induced sialidase activity inhibited by black seed oil ................................. 133
Figure 36. Ligand-induced sialidase activity inhibited by BSO component para-Cymene ...... 135
Figure 37. BSO and p-Cy do not inhibit TQ-induced sialidase activity .................................... 137
Figure 38. TQ-induced sialidase activity in the absence of TLR expression in HEK 293 cells . 139
Figure 39. Cell viability of TQ treated BMC-2 macrophage cells.................................................. 141
Figure 40. TQ-induced sialidase activity in non-metastatic infiltrating mouse mammary
adenocarcinoma (Sp1) cells ............................................................................................................. 143
**List of Abbreviations**

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Name</th>
</tr>
</thead>
<tbody>
<tr>
<td>4-MU</td>
<td>4-MUNANA, 2’-(4-methylumbelliferyl)-α-D-N-acetylneuraminic acid</td>
</tr>
<tr>
<td>BMC-2</td>
<td>Mouse bone marrow derived macrophage cell line</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>BSO</td>
<td>Black seed oil</td>
</tr>
<tr>
<td>cAMP</td>
<td>Cyclic adenosine monophosphate</td>
</tr>
<tr>
<td>cDNA</td>
<td>Complementary deoxyribonucleic acid</td>
</tr>
<tr>
<td>CC</td>
<td>Cold competitor – unlabeled NFκB oligonucleotide</td>
</tr>
<tr>
<td>CTX (-B)</td>
<td>Cholera toxin (subunit B)</td>
</tr>
<tr>
<td>DANA</td>
<td>2-deoxy-2,3-dehydro-N-acetylneuraminic acid</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>dsRNA</td>
<td>Double stranded ribonucleic acid</td>
</tr>
<tr>
<td>EBP</td>
<td>Elastin binding protein</td>
</tr>
<tr>
<td>GDP</td>
<td>Guanosine diphosphate</td>
</tr>
<tr>
<td>GPCR</td>
<td>G-protein coupled receptor</td>
</tr>
<tr>
<td>G-protein</td>
<td>Guanine nucleotide binding protein</td>
</tr>
<tr>
<td>GTP</td>
<td>Guanosine triphosphate</td>
</tr>
<tr>
<td>HEK293</td>
<td>Human embryonic kidney epithelial cell line</td>
</tr>
<tr>
<td>HEK-TLR4</td>
<td>HEK cell line stably transfected with human TLR4-MD2</td>
</tr>
<tr>
<td>HRP</td>
<td>Horse radish peroxidase</td>
</tr>
<tr>
<td>ICC</td>
<td>Immunocytochemistry</td>
</tr>
<tr>
<td>IκBα</td>
<td>Inhibitor of κappa B</td>
</tr>
<tr>
<td>IFN</td>
<td>Interferon</td>
</tr>
<tr>
<td>IgG</td>
<td>Immunoglobulin G</td>
</tr>
<tr>
<td>IKK</td>
<td>Inhibitor of IκB kinases</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>IL-1R</td>
<td>Interleukin 1 receptor</td>
</tr>
<tr>
<td>KD</td>
<td>Knock-down</td>
</tr>
<tr>
<td>KI</td>
<td>Knock-in</td>
</tr>
<tr>
<td>KO</td>
<td>Knock-out</td>
</tr>
<tr>
<td>LPS</td>
<td>Lipopolysaccharide</td>
</tr>
<tr>
<td>MAPK</td>
<td>Mitogen activated protein kinase</td>
</tr>
<tr>
<td>MD-2</td>
<td>Co-receptor for TLR4</td>
</tr>
<tr>
<td>MMP</td>
<td>Matrix Metalloproteinase</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger RNA</td>
</tr>
<tr>
<td>MYCO</td>
<td><em>Mycobacterium butyricum</em></td>
</tr>
<tr>
<td>MyD88</td>
<td>Myeloid differentiation primary response gene 88</td>
</tr>
<tr>
<td>Neu1-4</td>
<td>Neuraminidase 1-4 (sialidase)</td>
</tr>
<tr>
<td>NeuIn</td>
<td>Mice with Neu1 deficiency</td>
</tr>
<tr>
<td>NFκB</td>
<td>Nuclear transcription factor kappa B</td>
</tr>
<tr>
<td>PAMP</td>
<td>Pathogen associated molecular pattern</td>
</tr>
<tr>
<td>p-Cym</td>
<td>Para-Cymene</td>
</tr>
<tr>
<td>PTX</td>
<td>Pertussis toxin</td>
</tr>
<tr>
<td>SPI</td>
<td>Human breast cancer cell line</td>
</tr>
<tr>
<td>SRMN</td>
<td>Suramin</td>
</tr>
<tr>
<td>ssRNA</td>
<td>Single stranded ribonucleic acid</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>TBS</td>
<td>Tris buffered saline</td>
</tr>
<tr>
<td>TNF(R)</td>
<td>Tumor necrosis factor (receptor)</td>
</tr>
<tr>
<td>TS</td>
<td>Trans-sialidase</td>
</tr>
<tr>
<td>TQ</td>
<td>Thymoquinone</td>
</tr>
<tr>
<td>WT</td>
<td>Wild Type</td>
</tr>
</tbody>
</table>
Chapter 1

Introduction

1.1 *Nigella sativa* and Thymoquinone

The black seeds of the *Nigella sativa* plant have an extensive history of medicinal use that dates back thousands of years. In ancient Greece and Egypt, black seed oil was used by physicians to treat an assortment of illnesses including headaches, nasal congestion, toothaches and intestinal worms. *N. sativa*, a buttercup-like plant that belongs to the *Ranunculaceae* plant family, is cultivated around the world for its seeds which can be used as a spice or preservative agent. Today however, the seeds are primarily used for the extraction of an oil that is used in traditional medicine. Black seed oil (BSO) is thought to promote menstruation and increase milk production in women [1, 2]. Contemporary naturopathic medicine around the world has used BSO as a diuretic, carminative, treatment for asthma, bronchospasm, respiratory oppression, coughs, back pain, hypertension and obesity [1-3]. Until recently, few studies had been conducted to confirm the validity of the proposed medicinal value of BSO. In recent years, various studies have been conducted on BSO to investigate such properties as anti-microbial, hypotensive, anti-nociceptive, anti-histaminic, immunomodulatory, anti-inflammatory, anti-tumour, and anti-diabetic as well as many other characteristics [1].

Of the many constituents of BSO eluted by high pressure liquid chromatography, thymoquinone (TQ) has been shown to be one of the most active. Although present in only a small amount in the volatile oil portion of BSO, TQ has been associated with similar properties as those present in the crude oil such as anti-cancer, anti-diabetic and anti-inflammatory. TQ has been shown to produce many biological effects including disease treatment and prevention of
Thymoquinone is part of a structural group known as benzoquinones. Other benzoquinones, such as Aziridinyl Benzoquinone, are used as anti-neoplastic agents [4]. Thymoquinone is believed to share similar properties to the benzoquinones already in use as therapeutic drugs. It was the proclaimed anti-inflammatory properties that brought BSO and Thymoquinone to our lab for studies.

1.2 Inflammation

Inflammation is the cellular response to pathogen invasion that results from vascular dilation and an increased movement of immune cells into the affected area resulting in clinical appearance of swelling and reddening [5]. Many diseases originate from or are exacerbated by the inflammatory response such as chronic inflammation resulting in asthma, infections in the limbs of patients with diabetes and advancement of certain cancers. Thymoquinone may have a therapeutic role in inflammation, cancer and diabetes. The immune response is often manifested by the release of pro-inflammatory cytokines and chemokines from cells in the innate immune system. Cytokines and chemokines can work to activate immune cells or elicit a chemoattractive response in other cells in the vicinity. Cytokines such as IL-1 and TNF-α are markers of inflammation. Chemokines and adhesion molecules including MIP-1 and sICAM-1 are also primary indicators of inflammation. Th1- and Th2-dependent immune responses result in the production of various cytokines. When Th1 cells are activated, they produce pro-inflammatory cytokines such as IL-1, IL-2, IL-12, IFN-γ, and TNF-α that stimulate macrophages, Natural Killer cells, cytotoxic T cells and movement of other cells into the affected area [1, 6]. Whereas when the Th2 cells are activated, they stimulate B cell proliferation and antibody production[6]. In response to Th-2 mediated or humoral immunity, anti-inflammatory cytokines IL-4, IL-5, IL-10 and IL-13 are released [1, 6]. The ability of a substance to control the balance between Th1- and
Th2-associated releases of cytokines has been associated with both pro- and anti-inflammatory properties.

1.3 Toll-Like Receptors

Innate immune cells respond to pathogen invasion through cell surface receptors known as Toll-like receptors. Toll-like receptors (TLRs) are a family of pathogen recognition receptors that recognize pathogen-associated molecular patterns (PAMPs) from bacteria, viruses and fungi [7]. TLRs are responsible for mediating innate immune responses by stimulating the phagocytic properties of macrophage and dendritic cells as well as playing a major role in cytokine production and the subsequent activation of the cells of the adaptive immune system [8]. Macrophage and dendritic cells are classified as professional antigen presenting cells, which are known to produce many cytokines as well as link the innate and adaptive immune systems by the presentation of the antigens to activate T-cells. Hence, not only are TLRs crucial sensors of microbial infection, they also play an important role in the pathophysiology of infectious diseases, inflammatory diseases, and possibly in autoimmune diseases.

1.4 Sialidase

Sialidases are glycohydrolytic enzymes involved in activating cell surface receptors such as TrkA tyrosine kinase receptors and Toll-like receptors [9, 10]. There are four known mammalian sialidases; Neu1, Neu2, Neu3 and Neu4, which differ in their cellular location, function and substrate preference. Neu1 is located in the lysosome and at the cell surface; Neu2 is cytosolic, and Neu3 is the plasma membrane-bound form. Neu4, the most recently characterized sialidase, is found in various cellular locations like the inner lysosomal membrane and mitochondria [11-14]. Neu1 is found complexed with protective protein cathepsin A (PPCA) and either elastin binding protein, at the cell surface, or β-galactosidase (GBL1), in the lysosome
It has been suggested that cellular sialidases may be involved in the removal of sialic acid from receptors. There is evidence that this removal of sialic acid activates TrkA tyrosine kinase receptors allowing for dimerization, internalization and subsequent induction of the signaling cascade that results in cell differentiation or cell survival [9, 15]. Preliminary, unpublished data suggest that TLR activation may also be dependent on a similar cellular sialidase controlling mechanism that involves G-Protein Coupled Receptor (GPCR) and matrix metalloproteinase (MMP) activation [16]. The investigation of these factors has led us to believe that thymoquinone may modulate cellular sialidase activity through one or more of these components.

1.5 G-Protein Coupled Receptors

G-protein coupled receptors (GPCRs) are a large family of highly selective ligand activated transmembrane receptors that signal through heterotrimeric guanine nucleotide subunits known as $G_{α}$, $G_{β}$, and $G_{γ}$. GPCRs have been linked to TLR and sialidase activity [16]. Upon ligand binding, $G_{α}$ protein exchanges its GDP for GTP, dissociates from the $G_{β}$ and $G_{γ}$ subunits and acts as a second messenger type molecule [17]. GPCR and their corresponding G protein subunits play a major role in cell surface signaling across the plasma membrane [18]. Depending on ligand interaction and G protein association, GPCR may cause a highly focused cellular response or a broad spectrum response in many cell signaling pathways [19]. The response to ligand may also vary in different cell types. There are four major families of $G_{α}$ proteins, $G_{α}_{s}$, $G_{α}_{i}$, $G_{α}_{q/11}$, and $G_{α}_{12/13}$, which upon activation result in different cellular responses [20]. $G_{α}_{s}$ causes a broad spectrum response by inducing adenylyl cyclase 1-7 and 9 which results in the production of cyclic AMP (cAMP). The increase in cAMP activates protein kinase A to signal through its many pathways. $G_{α}_{i}$ inhibits all adenylyl cyclases except 2 and 4 to reduce the production of cAMP. Other subgroups of $G_{α}_{i}$ cause stimulation by reducing ATP to cAMP.
inhibition and by activation of PI3 kinase γ. Ga_{q/11} signals through the phosphoinositol pathway by activating membrane-bound phospholipase C to cleave PIP_{2} into IP3 and diacylglycerol activating protein kinase C. Ga_{12/13} controls cellular cytoskeleton remodeling and cellular migration through Rho family GTPase signaling [20]. This study explores the role of GPCR and associated guanine nucleotide subunits in TQ-induced cellular activity.

1.6 Matrix Metalloproteinase

Matrix metalloproteinase (MMP) enzymes have also been linked to sialidase and TLR activity [16]. MMPs are a family of zinc-dependent endopeptidases that have many functions. There are currently 23 known MMP family subgroups, most of which are responsible for restructuring the extracellular matrix by targeting multiple substrates like elastin, gelatin and collagen [21]. Some of the enzyme activities carried out by the various MMPs include elastase, gelatinase and collagenase. Previous research suggests that the most likely candidates to interact with GPCR and sialidase are those MMPs possessing elastase activity including MMP-2, -3, -7, -8, -9, and -12 [16].

1.7 Rationale, Objectives and Hypothesis

This study was conducted to test the proclaimed anti-inflammatory effects of black seed oil and thymoquinone in bone marrow derived mouse macrophage cells. Our work demonstrated that thymoquinone induced sialidase activity in a manner similar to bacterial lipopolysaccharide; subsequent research was aimed at elucidating the mechanism in which TQ induced sialidase activity. Further studies were conducted to discover the overall response to TQ in vitro and in vivo. By and large, we hypothesize that thymoquinone induces Neu4 sialidase activity through G-protein coupled receptor associated Ga, subunit and matrix metalloproteinase 9 to promote Toll-like receptor 4 activation and a pro-inflammatory response. This TQ-induced pathway leading to
a pro-inflammatory response may also be related to the previously discovered anti-cancer and anti-diabetic properties associated with TQ treatment. Therefore the objectives of this study were to:

1. Determine if thymoquinone is activating cellular sialidases.
2. Determine which sialidase thymoquinone is activating.
3. Determine if thymoquinone is acting in a pro- or anti-inflammatory manner.
5. Determine if thymoquinone is activating Toll-like receptors.
6. Link the observed properties of thymoquinone to the proclaimed anticancer and anti-diabetic properties.
Chapter 2
Literature Review

2.1 The Medicinal Properties of TQ

2.1.1 TQ & Cancer

Effects of thymoquinone on cancer cells

Promising anti-cancer activities have long been observed with thymoquinone treatment. Studies on thymoquinone suggest that it induces apoptosis and inhibits cellular growth in cancer cell lines [22]. The most clinically motivating property of thymoquinone treatment is that it is able to inhibit growth and induce apoptosis in multiple drug resistant cancer cell lines [22]. Thymoquinone is able to preferentially inhibit the growth of cancerous cells, such as osteosarcoma cells, and leave non-cancerous cells, such as primary mouse keratinocytes, with good viability and minimal damage [22]. Thymoquinone is cytotoxic towards fibrosarcoma, FsaR, and squamous cell carcinoma, SCCVII, cell lines but not towards normal fibroblast cell lines [23]. Since thymoquinone is nontoxic towards non-cancerous cells, it may be an alternative to the current drugs used today, which have prominent toxicity profiles. Regular doses of thymoquinone in water have been shown to prevent stomach tumours and fibrosarcomas in a methylcholanthrene-induced tumor model [22]. Mechanistically, the apoptotic and anti-cancer effects of thymoquinone have been categorized as either p53 dependent or p53 independent where apoptosis results by inhibiting DNA synthesis and/or the induction of cell cycle arrest [22, 24, 25].

p53 dependent effects of TQ

The transcription factor p53 plays a key role in tumour suppression and the prevention of genome mutations dubbing it the “guardian of the genome” [22]. p53 can perform many tasks in
the cell including activating DNA repair, inducing cell cycle arrest at G1, and initiating apoptosis [26]. Thymoquinone induces an unknown a molecular pathway involving p53 to induce apoptosis in certain cell lines. For example, in the human colon cancer cell line, HCT-116, thymoquinone treatment increases the mRNA and protein production of p53 and its target, p21WAF1 [24].

TQ may affect the ratio of apoptotic and anti-apoptotic factors Bax and Bcl-2 through p53 protein. Thymoquinone inhibits anti-apoptotic factor, Bcl-2, in p53 positive HCT-116 cells [24]. The Bcl-2 homolog, Bax, opposes Bcl-2 to induce cell apoptosis. The balance of the ratio between these homologs is important as it can result in cell survival or death. Therefore, thymoquinone pushes the Bcl-2/Bax ratio towards inducing apoptosis in cancerous cells [24].

*p53 independent effects of TQ*

*Caspase-induced apoptosis*

TQ treatment is able to induce a myriad of molecular pathways to cause apoptosis in physiologically important p53 independent manners as p53 is regularly mutated in patients with cancer [27]. p53 independent pathways are valuable in cells that do not express p53 such as the myeloblastic leukemia cell line, HL-60 [22]. In HL-60 cells thymoquinone activates Caspases 3 and 9 which cause a proteolytic cascade [22, 28]. The activation of Caspases 3 and 9 lead to structural protein and DNA degradation, and eventually to apoptosis. Thymoquinone also activates Caspase 8 in these cells to initiate further apoptosis and halt cytochrome C activation [28]. The inhibition of growth in these cells is also due to thymoquinone’s ability to disrupt the mitochondrial membrane [28]. Thymoquinone treatment also induced apoptosis through caspase activation in osteosarcoma cells and neoplastic keratinocytes [22].
**p16 inhibition**

The presence of another protein, p16, is often associated with poor prognosis when diagnosed with cancer[29]. p16 is a cyclin-dependent kinase that is most closely associated with cervical cancer because it has a compound effect with human papillomavirus to inactivate tumour suppressing retinoblastoma protein [29]. Thymoquinone treatment is able to reduce p16 protein to restore it to a healthy level and induce G1 phase cell cycle arrest in papilloma infected cells.

**cyclin B1 inhibition**

Mitosis promoting factor cyclin B1, complexed with Cdk1, increases during the cell cycle to induce mitosis when the cell is ready to divide [22]. Research shows that thymoquinone is able to decrease the amount of cyclin B1 in spindle cancer cells by an unknown mechanism. This deficiency in cyclin B1 causes cell cycle arrest at G2 before mitosis can begin. Therefore, thymoquinone can stop these cancerous cells from multiplying [22].

**Chemoprotective with standard drug therapies**

Cancer chemotherapy treatments are often drug concoctions made of the gold standard drugs available at the time of treatment. Thymoquinone has been studied clinically in conjunction with other cancer therapies such as isofosfamide, an alkylating agent and cisplatin, which cross links DNA and interferes with mitosis as well as doxorubicin that interacts with DNA through intercalation and inhibits protein synthesis [30-33]. When used in conjunction with other drug treatments, thymoquinone was able to reduce the toxicity of the other drugs by unknown mechanisms [22]. As described, thymoquinone has many qualities that may lead to new cancer treatments.
2.1.2 TQ & Diabetes

The incidence of diabetes mellitus has increased worldwide, with Asia showing one of the fastest rates of increase [34]. In recent years, medical sciences have seen a similar increase in research programs focused on novel treatments and a cure for diabetes. Thymoquinone has become a target of research as a potential natural drug to help treat diabetes [35]. Our research has hinted that some enzymes in the proposed TQ-induced molecular pathway of inflammation may also be linked to the insulin receptor and involved in the anti-diabetic effects seen after treatment with TQ.

Streptozotocin-induced model of type 1 diabetes mellitus

Diabetes research is often performed on mammalian models of chemically-induced diabetes. Streptozotocin is a chemical that is toxic towards insulin-producing pancreatic beta cells and induces a type 1 like diabetic state in models such as hamsters and rats [35]. Diabetic symptoms in these animals include increases in blood glucose and glycated haemoglobin levels, hyperglycemia and hypoinsulinemia [36, 37]. Thymoquinone treatment has been studied in both the hamster and rat models. The benefits observed were persistent for up to a month after stopping treatment [38, 39]. Thymoquinone treatment four weeks after streptozotocin injections in hamsters resulted in a significant reduction of blood glucose and glycated haemoglobin levels [38]. The observed benefits of thymoquinone in diabetic treatment may be due to its ability to decrease gluconeogenesis. The glucose level in hepatocytes tested 30 days post thymoquinone treatment remained lower than controls [38]. Studies in the rat model showed significant decreases in hyperglycemia and hypoinsulinemia when animals were co-treated with streptozotocin and thymoquinone [39]. Once again, these benefits were maintained for a month following treatment termination. Some studies associate beneficial effects of thymoquinone on
diabetes with an inhibition of the nitric oxide (NO) pathway. The NO pathway causes poor vascular function in patients suffering from diabetes [39]. Thymoquinone inhibits the action of both p44/42 and p38 mitogen-activated protein kinases involved with the NO pathway in a rat model with streptozotocin-induced diabetes mellitus [39]. Therefore, thymoquinone diminishes the negative effects of NO on vasculature in the diabetes disease state. Overall, the effects of Thymoquinone on blood glucose and NO levels reveal TQ as a promising new treatment for diabetes mellitus.

2.1.3 TQ & Inflammation

Although the medicinal properties of thymoquinone (TQ) have been extensively studied, there is a general consensus in the literature that the molecular pathway(s) of the anti-inflammatory effects of TQ are not well understood. Therefore most studies look at the major biological and cellular effects rather than molecular effects of TQ treatment on inflammation. The anti-inflammatory effects of thymoquinone have been tested in multiple study models including mouse models of allergic lung inflammation, LPS stimulated mast cells, rat models of adjuvant-induced arthritis and many more.

*Mouse models of allergic inflammation*

The anti-inflammatory properties of TQ were analyzed using an allergen-induced mouse model of bronchial asthma airway inflammation [40]. Mice were first sensitized with ovalbumin (OVA) antigen and subsequently challenged with the same antigen [41]. The antigen stimulation would create an increase in eosinophil and mast cells, mucus secretion and T-cell activation in the mice [41]. In studies where mice were pre-treated with intraperitoneal injections of TQ prior to being challenged with the OVA antigen, TQ was found to alter the balance of T-cell response by decreasing the Th2 response and shifting it towards a Th1 response [41, 42]. In essence, TQ
treatment shifted the response from humoral to cellular immunity as evidenced by a decrease in IL-4, IL-5 and IL-13 and an increase in IFN-γ production [41]. Studies using the mouse model of bronchial asthma also found that thymoquinone inhibited the generation of inflammatory indicators, thromboxane B2 and leukotriene B4 [41]. It is also believed that these properties of thymoquinone result from TQ targeting arachidonic acid metabolism, specifically the cyclooxygenase and 5-lipoxygenase pathways through an unknown mechanism [41].

*Adjuvant-induced model of arthritic inflammation*

Rheumatoid arthritis is an example of a systemic inflammatory disease. Experiments using a collagen-induced model of arthritis in rats showed that thymoquinone was able to reduce inflammation in the inter-phalangeal joints of arthritic rats. Treatment with TQ was also found to reduce soft tissue swelling and joint erosion as measured by arthographies. Collagen-induced production of pro-inflammatory cytokines, TNF-α and IL-1β, was reduced after TQ treatment in mice [43].

*Mast cell model of inflammation and NFκB*

Immune cell activation is typically associated with molecular pathways which influence transcription, translation and cytokine production. NFκB is a multiple subunit transcription regulator which is associated with cytokine production and inflammation. NFκB p50 subunit and p65 subunit homodimers are important in inflammation as they respond to pro-inflammatory signals and work in an opposing manner towards cytokine transcription where p50 blocks and p65 stimulates DNA promoters for cytokines [42]. Lipopolysaccharide (LPS) stimulation of the rat basophil cell line (RBL2H3) can be used as a mast cell model of inflammation since these cells resemble mucosal mast cells that mediate Th2 cell responses and contribute to chronic inflammation [42]. TNF-α production and NFκB activation are indicators of inflammation caused
by a Th1 cell response. LPS interacts with Toll-like receptor 4 (TLR4) to activate the NFκB signalling pathway to induce pro-inflammatory cytokine production and release. When treated with thymoquinone, there was a significant decrease in TNF-α mRNA production compared to LPS stimulated cells [42]. Therefore, there was a reduction in the LPS-induced Th1 immune response with thymoquinone treatment. Studies found that basophil cells treated with thymoquinone had the same amount of NFκB in the cytosol and nucleus as those cells treated with LPS. Conversely, with thymoquinone treatment there was an increase in the amount of NFκB p50 homodimer binding to the promoter to repress the genes for pro-inflammatory TNF-α [42]. This decrease in TNF-α expression correlated with protection from LPS-induced colitis. The reduction of TNF-α with thymoquinone treatment has also been observed in vivo [44][44][45][45][45][42].

Overall, previous studies have shown that Thymoquinone acts as an anti-inflammatory agent in cases of chronic inflammation and when used along with inflammation inducing lipopolysaccharide.

2.2 Sialidase and Inflammation

Thymoquinone may induce a pro-inflammatory response when used as a singular treatment. This inflammation may be induced through sialidase, TLR, GPCR and MMP.

2.2.1 Sialidase and TLR4

A role for sialidase enzymes in receptor activation was initially discovered in the ligand-induced activities of tyrosine kinase TrkA receptors. It was found that recombinant trans-sialidase derived from *Trypanosoma cruzi* was able to target TrkA receptors [15]. The deglycosylation of receptors by this sialidase proved to be important for the dimerization and activation of TrkA receptors. The sialidase activation upon ligand binding to TrkA, TLR and possibly other receptors
results in the hydrolysis of α-2,3 linked glycosyl sugar residues on the receptor, a key step in subsequent activation [9]. Recent data also suggest a similar role for cellular sialidase in the ligand-induced activation of Toll-like receptors, where an induction of Neu1 sialidase activity was observed upon bacterial lipopolysaccharide (LPS) binding to TLR4 [10]. It was found that Neu1 sialidase, either as part of the elastin receptor complex, or elsewhere on the membrane, was found in complex with TLR4 by co-immunoprecipitation [16]. Amith, S.R. et al also found that binding of bacterial lipopolysaccharide to TLR4 induced Neu1 sialidase activity. TLR4 activation, as measured by MyD88 co-localization, was markedly reduced in Neu1 deficient primary macrophage cells. Finally, in the absence of a TLR4 ligand, the activation of TLR4 was induced upon treatment with trans-sialidase.

2.2.2 Neu4 Sialidase

Neu4 is a novel sialidase enzyme discovered in 2003 [45]. Neu4 is ubiquitously expressed and has been found associated with the lysosome, mitochondria and various intracellular membrane components [11, 13, 46]. The DNA sequence of this sialidase gene is most homologous with plasma-membrane bound Neu3 [45]. Neu4 has a broad substrate specificity that may be beneficial in treating sialidase-related lysosomal storage disorders such as sialidosis, galactosialidosis and Tay-Sachs [12, 13]. The primary function and role of Neu4 has yet to be fully elucidated. Roles for Neu4 in the hydrolysis of gangliosides and regulation of lysosomal structure have been observed [12]. The knockout of Neu4 genes resulted in vacuolization and increased lysosomal storage in lung, spleen and macrophage cells. Neu4 may also play an important role in the desialylation of gangliosides in the brain, mainly the conversion of GD1 to GM1 [12]. Other problems resulting from the knockout of the Neu4 genes included increased levels of cholesterol, ceramide and polyunsaturated fatty acids [12]. A role for Neu4 in
malignant transformation has also been elucidated. In contrast to the role of Neu3, the presence of Neu4 shows a marked decrease in cancerous colon cells [47, 48]. Neu4 may also play a role in cancer though its ability to regulate apoptosis. Neu4 moderates the excessive accumulation of GD3 ganglioside in the mitochondria during the apoptotic response [11]. Transfection of Neu4 in human colon cancer cells up-regulated the apoptotic response and decreased cellular motility and invasion [47]. In general, this novel sialidase may play a role in both the inflammatory and anti-cancer properties associated with TQ treatment.

2.2.3 MMP, GPCR and Sialidase activity

*MMP9 and Sialidase activity*

Matrix metalloproteinase 9 (MMP9), which has significant elastase activity, is an important molecule in the inflammatory response and is secreted by neutrophils, macrophages and osteoclasts [49]. Elastin peptides, created from degrading elastin fibres with elastases such as MMP9 and leukocyte elastases during inflammation, are influential in pathological and physiological processes through binding to elastin binding protein (EBP) and the elastin receptor complex [50]. Elastin peptides can stimulate chemotaxis, cell proliferation and protease release. It has been shown that Neu1 is the sialidase enzyme present in the elastin receptor complex [51]. The EBP/elastin signal is then conveyed through sialidase activity carried out by the Neu1 portion of the complex [14].

*GPCR and MMP*

Recently, the role of MMPs in the pro-inflammatory response has been augmented to include their function in receptor trans-activation. The role of matrix metalloproteinase (MMP) in epidermal growth factor receptor (EGFR) activation has been vastly explored [52]. GPCR agonists, bombesin and lysophosphatidic acid (LPA), are able to induce the activation of EGFR in
the absence of epidermal growth factor ligand. It was discovered that the broad spectrum MMP inhibitor, galardin, was able to inhibit the trans activation of EGFR by bombesin and LPA [53]. Preliminary unpublished data (S.R. Amith, PhD Thesis: The role of Neu1 Sialidase in Toll-Like Receptor Activation) suggest a similar role for both GPCR and MMP in TLR4 activation [16]. Inhibitors to GPCR G proteins and MMPs are able to inhibit TLR4 ligand-induced sialidase activity and NFκB activation. Conversely, MMPs have also been found to activate certain GPCR such as the thrombin receptor, PAR-1 [54]. Elastin peptides, a product of the elastase activity of MMP, may influence proliferation of cells through different pathways such as the Gαi pathway of G-protein coupled receptors (GPCR) with tropoelastin, and Ras signalling pathway and opening L-type calcium channels, with elastin peptides [55, 56].

**GPCR and TLR4**

G proteins may play a role in both GPCR signaling and TLR signaling. Pertussis toxin (PTX) sensitive Gαi2 and Gαi3 protein regulate the signaling in selected GPCR and TLR interactions [57]. Treatment with PTX was able to reduce LPS-induced IL-1 production, p38 and ERK1/2 activation in macrophage cells. TLR4 binding by ligand LPS induced the phosphorylation of Gαi2 protein subunit. In mice lacking Gαi2 or Gαi1/3, there was a reduction in LPS-induced production of TNF-α, IL-10 and thromboxane B2 [57]. This demonstrates that G proteins play an important role in TLR signaling. Whether or not GPCR agonists are able to trans-activate TLRs in a similar manner to EGFRs has not been established.
Chapter 3

Materials and Methods

Cell Lines

BMC-2 cells are a stable line of mouse bone marrow derived macrophage cells acquired from Dr. Ken L. Rock, University of Massachusetts Medical School, Worcester, MA. HEK293 cells are a stable human embryonic kidney epithelial cell line obtained from Dr. Leda Raptis, Department of Microbiology and Immunology, Queen’s University, Kinston, Ontario, Canada. Both cell lines were grown in 1x DMEM medium (Gibco, Rockville, MD, USA) containing 5% horse serum (Gibco) and 3% fetal calf serum (HyClone, Logan, Utah, USA) or 5% fetal calf serum at 37°C in 5% carbon dioxide.

HEK-TLR4/MD2 cells are from a HEK parental cell line that has been stably transfected with human TLR4 gene in a pCDNA3 expression vector and human MD2 gene in an expression plasmid. These cells were a kind gift from Prof. Rudi Beyaert, Unit for Molecular Signal Transduction in Inflammation, Department for Molecular Biomedical Research, VIB; and Department for Molecular Biology, Ghent University, Technologiepark 927, B-9052 Zwijnaarde, Belgium. Cells were grown in 1x DMEM medium (Gibco) containing 5% fetal calf serum (HyClone) and 0.8μg/ml G418 at 37°C in 5% carbon dioxide.

1140F01 cells are Neu1 deficient human fibroblast cells that were isolated from patients with lysosomal storage disorder sialidosis and immortalized [58]. These cells were a kind gift from Dr. Alexey V. Pshezhetsky, Department of Pediatrics and Biochemistry, Montreal University, Service de Genetique, Ste-Justine Hospital, 3175 Cote-Ste-Catherine, H3T1C5,
Montreal, QC, Canada. Cells were grown in 1x DMEM medium (Gibco) containing 5% horse serum (Gibco) and 3% fetal calf serum (HyClone) and passaged with 1x Trypsin-EDTA.

SP1 cells are a non-metastatic infiltrating mouse mammary adenocarcinoma cell line provided by Dr. Bruce Elliott, Pathology and Molecular Medicine, Queen’s University, Kingston, Ontario, Canada [59]. Cells were grown in DMEM (Gibco) medium containing 5-10% fetal calf serum (HyClone) at 37°C in 5% carbon dioxide.

**Mouse Models**

Wild Type, NeuIn and Neu4 knockout mice were obtained from Alexey Pshezhetsky, Departments of Pediatrics and Biochemistry, Montreal University, Service de Genetique, Ste-Justine Hospital, Montreal, QC, Canada.

A true knock out of the Neu1 gene is lethal therefore a partial knock down of Neu1 is required to study this enzyme [60]. The knockdown of the Neu1 was accomplished in the NeuIn mice with a hypomorphic cathepsin A that had a secondary reduction of the Neu1 activity (Neu1-CathA KD) [60]. Approximately 90% of the Neu1 enzymes in the NeuIn mice were inactive.

Neu4KO was achieved by injecting pBluescript vector containing KO genes into R1 ES cells which were subsequently injected into C57BL/6H blastocysts and implanted into pseudo pregnant female mice. Male offspring were then crossed with C57BL/6J mice. The F2 offspring were identified by the agouti coat and genotyped with southern blot analysis [12].

**Primary mouse bone marrow derived macrophage cells**

Bone marrow was flushed from the femur and tibia of wild type, NeuIn, and Neu4KO mice. Cells were spun at 900rpm for 3 minutes and resuspended in red cell lysis buffer for 5 min at room temperature. Cells were washed with sterile 1x TBS and plated in 6, 12 or 24 well plates. Cells
were grown in RPMI 1640 medium (Gibco) containing 10% heat inactivated fetal calf serum, 20% monocyte colony stimulating factor (M-CSF) and 1x L-glutamine-penicillin-streptomycin (Pen/Strep, Sigma-Aldrich Canada Ltd., Oakville, Ontario) for 7-10 days at 37°C in 5% carbon dioxide. Primary bone marrow macrophage cells are 95% positive for F4/80 marker by day 7 as determined by flow cytometry [61]. 40000 or more cells were required to examine on an Epics XL-MCL flow cytometer (Beckman Coulter Miami, FL) and analyzed with Expo32 ADC software (Beckman Coulter, Miami, FL).

**Ligands**

Thymoquinone (TQ, 99%, Aldrich, St. Louis, MO, USA) was reconstituted into solution by mixing with 57% dimethyl sulfoxide (DMSO, Bio Shop Canada Inc., Burlington, ON, Canada) until dissolved. 1x Tris buffered saline (TBS, Tris from Bio Shop Canada Inc.) was then added to the solution. Lipopolysaccharide (LPS, purified by phenol extraction from *Serratia marcescens*) was used as a TLR4/MD2 ligand at concentrations ranging from 5μg/ml to 5 mg/ml. Bombesin acetate hydrate (Sigma-Aldrich, Oakville, Canada) was used as a GPCR ligand at 500μg/ml. Killed *Mycobacterium butyricum* (DIFCO) was used as a TLR2 ligand at 10mg/ml.

**Inhibitors**

Tamiflu (oseltamavir phosphate, Hoffman-La Roche Ltd., Mississauga, Ontario, Lot # BS00060168) was used as a broad range neuraminidase inhibitor (Figure 1A.). DANA (2-deoxy-2,3-dehydro-D-N-acetylneuraminic acid), is an inhibitor of sialidases (Figure 1B) [62]. DANA is able to inhibit Neu2 and Neu3 at low concentrations and may inhibit other sialidase enzymes at higher concentrations. DANA exhibits sialidase inhibition by binding to the active site of the enzyme [62]. Cholera toxin subunit B (CTX-B) acts as an inhibitor of Neu3 by binding the Neu3-specific substrate, GM1 ganglioside (Figure 1C.) [63]. Galardin (GALA, GM 6001, Calbiochem-
Figure 1. Structural diagrams of inhibitors

This figure depicts the structure of the inhibitors used throughout this study.

A) Structure of Tamiflu
B) Structure of 2-deoxy-2,3-dehydro-D-N-neuraminic acid (DANA) [64]
C) Structure of Cholera Toxin (CTX) (illustration by David S. Goodsell of The Scripps Institute). Crystal structure of CTX subunit B [65]
D) Structure of Galardin [66]
E) Structure of Piperazine (MMPII Inhibitor, Calbiochem-EMD Chemicals Inc.)
F) Crystal structure of Pertussis Toxin (PTX) [67]
G) Structure of Suramin
H) Structure of K252a [68]
I) Structure of para-Cymene
A  
Tamiflu

B  
2-deoxy-2,3-dehydro-D-N-acetyleneuraminic acid

C  
CTX B Subunit

D  
Galardin

E  
Piperazine

F  
Pertussis Toxin

G  
Suramin

H  
K252a

I  
para-Cymene
EMD Chemicals Inc., Darmstadt, Germany) was used as a broad spectrum cell-permeable inhibitor of MMP 1 (IC50=400pM), 2 (IC50=500pM), 3 (IC50=27nM), 8 (IC50=100pM) and 9 (IC50=200pM). Galardin inhibits MMPs by forming a complex with the zinc in the active site of these enzymes (Figure 1D.) [66]. Piperazine (PIPZ, MMPII inhibitor, Calbiochem-EMD Chemicals Inc.) was also used as a broad range inhibitor MMP, specifically MMP1 (IC50=24nM), MMP3 (IC50=18.4nM), MMP7 (IC50=30nM), and MMP9 (IC50=2.7nM) (Figure 1E). Pertussis toxin (PTX, from Bordatella pertussis, 99% pure, Aldrich St. Louis, MO USA) was used as an inhibitor of the Gαi subunit of GPCR (Figure 1F.) [20]. PTX causes structural changes in the Gαi proteins [69]. Suramin (suramin sodium salt, Sigma-Aldrich) uncouples the G proteins from the GPCR receptor complex (Figure 1G.). Other inhibitors of GPCR included cholera toxin (CTX, from Vibro cholera, Sigma) which permanently ribosylates the Gαs subunit (Figure 1C.) [63]. Inhibitor K252a (from Nocariopsis species, Calbiochem, La Jolla, CA, USA) was utilized to inhibit tyrosine protein kinase activity inhibitor in Trk receptors by inhibiting tyrosine phosphorylation of TrkA (Figure 1H.) [68]. Nigella sativa components black seed oil (BSO, Expolanka Commodities, Sri Lanka) and para-Cymene (p-Cy, 99% pure, Aldrich, St. Louis, MO, USA) were tested as inhibitors of ligand-induced sialidase activity (Figure 1I.).

**Antibodies**

Rabbit anti-human Neu1 IgG, mouse anti-human Neu2 IgG, rabbit anti-mouse TLR4 IgG, rat anti-human MyD88, and rabbit polyclonal anti-MMP9, were all acquired from Santa Cruz Biotechnology, Santa Cruz, CA, USA. The rabbit IgG antibodies against NFκBp65 (RelA), phospho-specific NFκBp65 (RelA) pS529 and IκBα were purchased from Rockland Inc., Gilbertsville, PA, USA. Antibodies against neuraminidase, Neu1-4 and MMP9 had neutralizing effects on the target proteins. This included the mouse anti-human Neu3 IgG (Medical &
Biological Laboratories Co., Ltd., Japan) and rabbit anti-human Neu4 IgG (Proteintech Group Inc., Chicago, IL, USA). AlexaFluor-labeled secondary antibodies included goat anti-rabbit AlexaFluor 488 (Molecular Probes, Eugene, OR, USA), goat anti-rabbit AlexaFluor 594 (Molecular Probes), goat anti-mouse 594 (Invitrogen, Corp.), and rabbit anti-rat AlexaFluor 488 (Invitrogen). Horse radish peroxidase-labeled goat anti-rabbit antibody was obtained from Santa Cruz Biotechnology.

**Live Cell Sialidase Activity - Sialidase Assay**

Cells were grown overnight on 12mm circular glass slides in DMEM medium containing 5% fetal calf serum (BMC2, HEK293, SP1) or 5% fetal calf serum and 0.8mg G418 (HEK-TLR4) at 37°C in 5% carbon dioxide. After removal of medium, 2μl of 0.2mM 4-MUNANA (4-MU) substrate [2’-(4-methylyumbelliferyl)-α-D-N-acetylneuraminic acid] (Sigma) in Tris buffered saline pH 7.4 was added with 2μl mounting medium (Dako) to cells alone (Control), with 2μl of ligand or with 2μl of inhibitor and 2μl of ligand. Inhibitors were either used directly with substrate and ligand or used to pre-treat the cells for 5 minutes. The substrate was hydrolyzed by sialidase to give free 4-methylumbelliferone which has a fluorescent emission at 450 nm (blue color) following excitation at 365 nm. Fluorescent images were taken at one minute time intervals after adding substrate using epi-fluorescent microscopy (40X objective). Images were analyzed for mean fluorescence using ImageJ version 1.38x software. Red, green and blue were measured for 50 points surrounding the cells in each image. Data were assembled and one way ANOVA tests were completed using GraphPad Prism version 5 software.
**Sialidase Standardization**

Standardization of the sialidase assay was completed by adding 2μl of 4-MU substrate with 2μl DAKO mounting medium and various concentrations of neuraminidase (from *Clostridium perfringens*) to a glass slide. The substrate was hydrolyzed by sialidase to give free 4-methylumbelliferone which has a fluorescent emission at 450nm (blue color) following excitation at 365nm. Fluorescent images were taken at one minute time intervals after adding substrate using epi-fluorescent microscopy (40X objective). Images were analyzed for mean fluorescence using ImageJ version 1.38x software. Red, green and blue were measured for 50 points on each picture. Data were assembled using GraphPad Prism version 5 software.

**Immunocytochemistry of anti-Neu and anti-MMP9 antibodies**

Cells were grown for 48 hours on 12mm circular glass slides in DMEM medium containing 5% fetal calf serum (BMC-2) or RPMI medium containing 10% fetal calf serum, 20% MCSF and 1x Pen/Strep (primary macrophage cells) at 37°C in 5% carbon dioxide. After removal of medium, cells were fixed with 4% paraformaldehyde for 30 minutes and left non-permeabilized or permeabilized with 0.2% Triton-X in TBS for 5 minutes. Cells were treated with 1μg/ml of either rabbit anti-human Neu1 antibody, mouse anti-human Neu2 antibody, mouse anti-human Neu3 antibody, 1μg/ml rabbit anti-human Neu4 antibody, or 1μg/ml rabbit anti-human MMP9 antibody for 60 minutes at 37°C. Cells were then washed and treated with one of two secondary antibodies, goat anti-rabbit IgG or goat anti-mouse IgG labeled with AlexaFluor 488 (BMC-2) or AlexaFluor 594 (primary macrophage cells) for 60 minutes at 37°C. Fluorescent images were taken using epi-fluorescent microscopy (40X objective).
**Immunocytochemistry of NFκB**

Cells were grown for 48 hours on 12mm circular glass slides in DMEM medium containing 5% fetal calf serum (BMC-2) or 5% fetal calf serum and 0.8μg/ml G418 (HEK-TLR4) at 37°C in 5% carbon dioxide. Cells were left untreated (control and ligands only) or treated with 50μl of inhibitors for 30 minutes at 37°C followed by treatment with 50μl of ligands at different time intervals ranging from 1 to 30 minutes at 37°C. Cells were fixed with 4% paraformaldehyde for 30 minutes on ice and permeabilized with 0.2% TritonX in TBS for 5 minutes on ice. Cells were treated with either 20μl 1:200 rabbit anti-NFκBp65 or 20μl 1:200 rabbit anti-IκBα for 60 minutes at 37°C. Cells were washed and treated with 20μl 1:700 goat anti-rabbit Alexa 594 for 60 minutes at 37°C. Fluorescent images were taken using epi-fluorescent microscopy (40X objective).

**TLR4 co-localization with MyD88**

Primary macrophage cells were grown for 7-10 days on 12mm circular glass slides in RPMI medium containing 5% fetal calf serum, 20% MCSF and 1x Pen/Strep at 37°C in 5% carbon dioxide. Cells were left untreated (control and ligands only) or treated with 50μl of inhibitors for 30 minutes at 37°C followed by treatment with 50μl of ligands for 5 minutes at 37°C. Cells were fixed with 4% paraformaldehyde for 30 minutes on ice and permeabilized with 0.2% TritonX in TBS for 5 minutes on ice. Cells were treated with 20μl of antibody cocktail containing 1:200 rabbit anti-MyD88 IgG and 1:200 rat anti-TLR4/MD2 IgG for 1 hour at 37°C. Cells were washed and treated with 20μl of 1:700 goat anti-rabbit AlexaFluor 594 antibody for 1 hour at 37°C. Cells were then washed and treated with 20μl of 1:700 rabbit anti-rat AlexaFluor
488 antibody for 1 hour at 37°C. Fluorescent images were taken using epi-fluorescent microscopy (40X objective).

**Cell Lysates**

Cultured HEK-TLR4 cells were left untreated or medium was removed and 200μM Tamiflu was added for 1 hour at 37°C. Tamiflu was removed and cells were treated with 250μg/ml TQ for 10 minutes at 37°C. Medium and treatments were removed and cells were washed with sterile 1x TBS before being spun at 2500rpm for 10 minutes at 5°C. Cells were incubated in 200μl of lysis buffer containing 50mM Tris, pH 8, 150mM NaCl, 1% NP-40, 0.2mg/ml leupeptin, 1% β-mercaptoethanol, and 1mM phenylmethanesulfonyl fluoride (PMSF) on ice for 30 minutes. Cells were centrifuged at 13000rpm for 20 minutes at 4°C. Supernatants containing cell lysates were removed and stored at -80°C.

**Nuclear extracts**

BMC-2 or HEK-TLR4 cells were incubated overnight in serum-free 1x DMEM medium at 37°C in 5% carbon dioxide. Cells were left in serum free medium or medium was replaced by 200nM Tamiflu, 500ng/ml Galardin, or 50ng/ml pertussis toxin for 30 minutes to 1 hour at 37°C. Inhibitors were replaced with 100μg/ml to 500μg/ml thymoquinone for 1 minute to 1 hour at 37°C. Treatments and medium were removed from cells and replaced with 5ml of sterile 1x TBS or spun at 2500rpm for 5 minutes at 4°C before removing supernatant. Nuclear contents were then extracted from cells in one of two manners, with self-made buffers or with Ner-Per Nuclear and Cytoplasmic Extraction Reagents kit from Pierce Biotechnology, Rockford, IL, USA. The first method involved incubating cells in 1ml of Buffer A containing 100mM HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid), 100mM KCl, 100mM EDTA (ethylenediamine tetraacetic acid), 100mM DTT (dithiothreitol), protease inhibitors and 10% NP-40 (Nonidet-P40)
on ice for 10 minutes. This was followed by spinning the samples at 13000rpm for 5 minutes at 4°C. Supernatant was removed and discarded in most cases as it contained the cytosolic fraction. Samples were then resuspended in 150μl of Buffer B containing 100mM HEPES, 2M NaCl, 5mM EDTA and 50% glycerol, and incubated on ice for 2 hours. Samples were centrifuged at 13000rpm for 5 minutes at 4°C and supernatant containing the nuclear fraction was stored at -80°C.

After cells were treated and washed, using the Pierce kit, cells were incubated with 200μl of CERI solution diluted with 1:00 protease inhibitor cocktail on ice for 10 minutes. While remaining on ice, 11μl of CERII solution was added to the samples for 1 minute. Cells were spun at 13000rpm for 5 minutes at 4°C. After removal of cytosolic fraction in the supernatant, 100μl of NER solution diluted with 1:100 protease inhibitor cocktail was added to the sample and incubated on ice for 40 minutes. Samples were vortexed for 15 seconds every 10 minutes. Samples were centrifuged at 13000rpm for 10 minutes at 4°C. Supernatant containing the nuclear fraction was stored at -80°C.

**Bradford assay**

Bovine serum albumin (BSA, Sigma, USA) stock at 10mg/ml was diluted 1:50 with distilled water and used to create a standard curve of 1, 2, 4, 8, and 16μg. Distilled water was added to BSA dilutions and 1-2μl of each sample to get a total volume of 800μl. 200μl of Bradford reagent (Sigma-Aldrich, USA) was added to BSA dilutions and each sample for 30 minutes. Absorbance was measured at 595nm. Data were assembled using GraphPad Prism version 4 software. Protein concentration in samples was determined from standard curve analysis.
**Electrophoretic mobility shift assay (EMSA)**

Nuclear extracts were used to assess the binding of NFκB to DNA. Extract volumes containing 8-20μg of sample were treated with 5ng of biotinylated NFκB probe (5’-AGT TGA GGG GAC TTT CCC AGG-3’, Operon Biotechnologies Inc. Huntsville, AL, USA) and Pierce Lightshift Chemiluminescent EMSA kit (Rockford, USA) contents according to protocol at room temperature for 30 minutes. Extracts were run on an 8% acrylamide gel at 70-100V and transferred to nylon membrane (Millipore) at 100mA on an Owl semi dry blotter for 20 minutes. Membrane was cross linked using UV radiation on a Spectroline cross linker on optimized setting. The membrane was probed with 7.5-20μl of Streptavidin-HRP in 7.5 ml blocking buffer (Pierce ESMA kit) for 15 minutes at room temperature. Results were detected using chemiluminescent substrate (Pierce EMSA kit) and X-ray film.

**Western Blot Analysis**

HEK-TLR4 cell lysate or nuclear extract, or primary macrophage cell lysate samples containing 12μg to 20μg of protein were boiled with sample buffer containing 0.5M Tris HCl, Glycerol, 10% SDS, 0.5% Bromophenol blue and 5% β-mercaptoethanol, and distilled water or lysis buffer for 10 minutes at 80-90°C. Molecular weight marker was boiled for 5 minutes at 80-90°C. Samples were run on an 8% acrylamide SDS-PAGE gel at 80-100V and transferred to a PVDF membrane (Millipore) at 100mA on an Owl semi-dry blotter for 1 hour. Membrane was blocked with 5% BSA in Tween-TBS (Tween 20, polycyoxethylene sorbitan monolaurate, Sigma) for 2 to 3 hours. Phospho specific anti-NFκBp65 antibody was used to probe the blot at 1:1000 to 1:5000 dilutions in 5% BSA/TTBS over night at 4°C. The membrane was incubated with secondary goat anti-rabbit HRP labeled antibody at 1:10,000 dilution in 5%BSA/TTBS for 1
hour at room temperature. Results were detected using chemiluminescent substrate (Western chemiluminescent detection kit, Santa Cruz) and X-ray film.

**mRNA extracts and polymerase chain reaction (PCR)**

Cells were left untreated (control) or treated with ligands for 2, 4, 6, or 8 hours at 37°C. Cells were spun at 900rpm for 10 minutes to form pellets. Pellets were resuspended in 1ml of TRI reagent (Ambion, Austin, TX, USA) for 5 minutes at room temperature. Samples were treated with 200μl of chloroform for 2-15 minutes at room temperature. Samples were spun at 12000xg for 15 minutes at 4°C. Aqueous layer containing RNA was transferred to new tubes and 500μl of isopropanol was added to each tube for 5-10 minutes at room temperature. Samples were spun at 12000xg for 8 minutes at 4°C. Ethanol (1ml of 70%) was added to each pellet and samples were spun at 7500xg for 5 min at 4°C. Ethanol was discarded and pellets were air dried for 5 minutes. Pellets were resuspended in 50μl of RNase free water and incubated for 10 minutes at 55-60°C. RNA was measured using ND-1000 micro-spectrophotometer.

**Reverse Transcription – Polymerase Chain Reaction**

RNA was diluted to 250μg/ml. Master mix was made containing 1μl of random primers (Invitrogen Corp., Browndeer, WI, USA), 1μl dNTP mix, 2μl 5x Buffer FS, 1μl RNase inhibitor (RNase Out Recombinant Ribonuclease inhibitor, Invitrogen Corp.) and 9.5μl RNase free water per sample was prepared. Master mix was added to 1μg of RNA and 0.5μl of reverse transcriptase (RT) enzyme (M-MLV RT, Invitrogen Corp.). Samples were cycled on the thermocycler (Thermo Electron Corporation) on a reverse transcriptase program.
Primers were diluted in RNase free water according to specifications. Primers used were acquired from Integrated DNA Technology, Coralville, IA include:

- **β-Actin**: (660bp) Forward 5’-TGA CGG GGT CAC CCA CAC TGT GCC CAT-3’
  Reverse 5’-CTA GAA GCA TTT GCC GAC GAT GGA GGG-3’
- **IL-6**: (295bp) Forward 5’-GAA CTC CTT CTC CAC AAG CG-3’
  Reverse 5’-GAA TCC AGA TTG GAA GCA TCC-3’
- **TNF-α**: (413bp) Forward 5’-GAG TGA CAA GCC TGT AGC CCA TGT TGT AGC-3’
  Reverse 5’-GGC AAT GAT GAT CCC AAA GTA GAC CTG CCC AGA CT-3’
- **MIP-1β**: (214bp) Forward 5’-TGT CTC TCC TCA TGC TGA TG-3’
  Reverse 5’-GTA CTC CTG GAC CCA GGA T-3’

Diluted primers were then added to 2μl of cDNA and 3μl of 5x Taq Polymerase Master Mix (New England BioLabs Inc.). DNA mixture was placed in the thermocycler (Thermo Electron Corporation) on a cycling program dependent on primer.

10μg of samples were loaded and run on 1.2% agarose gels containing 10μl of Ethidium Bromide. Along with the samples, 100 base pair ladder (Invitrogen) was run on the agarose gels. Gels were run at 55V to 75V. Gels were analyzed with Fluorchem HD2 Imaging System (Alpha Immunotech, San Leandro, CA, USA).

**Cytokine array profiling**

Blood was collected from wild type, Neu1 deficient (NeuIn) and Neu4 knockout (Neu4KO) mice before (control) and 5 hours after intraperitoneal treatment with 2mg/kg thymoquinone. Serum was extracted from blood and used for cytokine analysis with R&D System Array Profiling kit. All reagents were used at room temperature. Antibody cocktail was reconstituted in 100μl of sterile distilled water and stored at 4°C. The membrane was blocked with 2ml of blocking buffer at room temperature for 1 hour. Sera were prepared with buffers and
15μl of antibody detection cocktail as per kit protocol. Sample mixture was incubated at room temperature for 1 hour. After removal of blocking buffer, sample mixture was added to membranes and incubated overnight at 4°C. Sample antibody mixture was removed and membrane was washed. Streptavidin-HRP was diluted to 1:2000 with kit buffer and 1.5ml was added to each membrane for 30 minutes at room temperature. Chemiluminescent detection reagents (Pierce) were used and membranes were analyzed with Fluorchem HD2 Imaging System (Alpha Immunotech, San Leandro, CA, USA).

**Cell Viability**

HEK-TLR4 cells were grown overnight in 24 well plates on 12mm circular glass cover slides in DMEM medium containing 5% fetal bovine serum and 0.8μg/ml G418 at 37°C in 5% carbon dioxide. After removal of medium, 50μl of 200nM Tamiflu was added to the cells for 1 hour at 37°C. Tamiflu was removed and cells were left untreated (control) or treated with 100μl of 500μM or 1mM hydrogen peroxide (H₂O₂), or 100μl of 500μg/ml, 250μg/ml or 100μg/ml thymoquinone for 1 hour at 37°C. Stain mixture was prepared using 1μl of Acridine orange and 1μl of Propidium iodide solution (Sigma-Aldrich) in 998μl of sterile 1x TBS. To stain and mount cells, 1μl of stain mixture was used with 4μl of DAKO mounting medium. This stain combination caused nonviable cells to appear red and viable cells to appear green in colour. Cells were viewed and pictures were taken using epi-fluorescent microscopy.

**Cell Morphology**

BMC-2 cells were grown for 6 hours on 24 well plates in DMEM medium containing 5% fetal bovine serum at 37°C in 5% carbon dioxide. Medium was not removed from control cells. After removal of medium from the cells requiring treatment, either 80μl of 1mg/ml or 200nM Tamiflu, or 100μl of 100μg/ml TQ was added to the wells. Pictures were taken immediately on
Fisher Scientific inverted microscope under phase contrast using Micron camera software. Cells were returned to the 37°C incubator and removed to take pictures every 10 minutes. After 30 minutes of treatment, Tamiflu was removed and replaced by 100μl of 100μg/ml TQ.
Chapter 4  
Results

4.1 TQ activates sialidase in macrophage cells

4.1.1 Quantification of sialidase activity

Quantification of the sialidase enzymatic activity in the live cell sialidase assay was required to estimate the amount of active enzyme units in each live cell sialidase assay. A standard curve of fluorescence was obtained using commercially available neuraminidase enzyme (from *Clostridium perfringens*) at known enzyme unit concentrations with 4-MUNANA substrate. Fluorescence was measured by taking the red, green and blue channel measurements at fifty points in each picture and analyzed to create a standard curve of enzyme activity in relation to mean fluorescence emitted (Figure 2.). Controls were completed for this assay to ensure that the blue fluorescence was due to the activation of sialidase enzymes including the use of neuraminidase (from *C. perfringens*) alone, 4-MUNANA with and without cells. The use of this assay was established by Amith et al. in 2009 [10].

4.1.2 Black seed oil and component para-Cymene inhibit ligand-induced sialidase activation

Previously we have shown that sialidase activity is associated with ligand-induced receptor activation in live cells. This sialidase enzyme activity was found to mediate the activation of such receptors as tyrosine kinase TrkA and Toll-like receptors (TLR) [9, 16]. Treatment of live macrophage cells with pro-inflammatory ligands, such as lipopolysaccharide (LPS) a TLR4 ligand and Bombesin (Bomb) a GPCR agonist, caused an activation of sialidase 1-3 minutes following treatment. The sialidase activity was found in the periphery of the cells using a fluorogenic sialidase specific substrate, 4-MUNANA (4-MU, [2’-(4-methylumbelliferyl)-α-D-
Figure 2. Standardization of enzyme activity in live cells sialidase assay performed with *C. perfringens* derived neuraminidase

Sialidase specific substrate 4-MUNANA was added alone (control), or with a standard curve of Neuraminidase derived from *Clostridium perfringens* from 10 enzyme units(U)/ml to 0.109U/ml to a glass slide. The free 4-methylumbelliferone fluorescence was measured at 450nm (blue color) following excitation at 365nm. Fluorescent images were acquired in triplicate 1 minute after treatment using epi-fluorescent microscopy (40X objective). Mean fluorescence of the images was acquired using ImageJ version 1.38x software and analyzed using Graph Pad Prism 5 software. Top graph depicts three individual experiments whereas the bottom graph represents the average of the three experiments.
N-acetylneuraminic acid], which fluoresces at 450nm upon hydrolysis by sialidase enzymes. Cells were viewed and pictures were taken using epi-fluorescence microscopy. The fluorescence levels were measured in each image by taking the average of the red, green and blue channels at fifty points surrounding the cells. We believe that anti-inflammatory agents would be able to inhibit the sialidase activity induced by these ligands. To determine if the anti-inflammatory natural medicinal compound Black seed oil (BSO) and its component para-Cymene (p-Cy) were able to inhibit the sialidase response, they were tested for the ability to inhibit ligand-induced sialidase activity in live macrophage cells treated with either LPS or Bombesin. Both BSO and p-Cy were able to inhibit the ligand-induced sialidase activation (Appendix A Figure 35. And Appendix A Figure 36.).

4.1.3 Black seed oil component Thymoquinone induces sialidase activity

Since thymoquinone is an active component of BSO and has been found to have anti-inflammatory properties [41, 42], we originally hypothesized that TQ would inhibit LPS-induced sialidase activity in live macrophage cells. TQ was tested for its ability to inhibit ligand-induced sialidase activity in live BMC-2 macrophage cells using the sialidase specific fluorogenic substrate, 4-MU. Contrary to our hypothesis, TQ did not inhibit LPS-induced sialidase activity in live BMC-2 macrophages (Appendix A Figure 37.) Unexpectedly, TQ itself induced sialidase activation at concentrations as low as 1μg/ml in live BMC-2 cells (Appendix A Figure 38. and Figure 3.).

TQ in solution is mixed with 57% dimethyl sulfoxide (DMSO) in 1x Tris buffered saline (TBS) as experimentally determined. As a control, to ensure that TQ was responsible for the sialidase activation, live cell sialidase assays were performed with DMSO alone and 57% DMSO and TBS. Neither DMSO nor DMSO in TBS was able to induce sialidase activity in BMC-2 cells
Figure 3. Dose dependent TQ-induced sialidase activity in BMC-2 macrophage cells

BMC-2 cells were incubated overnight on 12mm circular glass slides in DMEM medium containing 5% fetal calf serum at 37°C. After removal of medium, 0.2mM 4-MUNANA (4-MU) substrate [2’-(4-methylumbelliferyl)-α-D-N-acetylneuraminic acid] (Sigma) in Tris buffered saline pH 7.4 was added with mounting medium (Dako) to cells alone (Control), with 11.2mg/ml killed *Mycobacterium butyricum* (MYCO), or thymoquinone (TQ) in 57% DMSO in TBS at concentrations of 1mg/ml to 100ng/ml. The free 4-methylumbelliferone fluorescence was measured at 450nm (blue color) following excitation at 365nm. Fluorescent images were taken at one minute time intervals after adding substrate using epi-fluorescent microscopy (40X objective). Fluorescence in pictures was analyzed by averaging the red, green and blue channels of 50 points proximal to cells using ImageJ software. Mean fluorescence was graphed using GraphPad Prism software. *P* values represent significant differences at 95% confidence using the Bonferroni’s multiple comparison tests to compare all treatments. These data represent one of ten repeated experiments.
(Figure 4A.). To determine if TQ had its own sialidase activity or was inducing the sialidase activity in BMC-2 cells, sialidase assays were performed using TQ and 4-MUNANA (4-MU) without cells. As a control to ensure that sialidase enzymes were able to cleave 4-MUNANA to release 4-methylumbelliferone and cause the emission of a blue fluorescence, neuraminidase extracted from *C. perfringens* was tested with 4-MUNANA in the absence of cells. *C. perfringens* neuraminidase was able to induce extensive blue fluorescence. This control was repeated using TQ treatment with 4-MUNANA alone which did not result in blue fluorescence and therefore did not possess sialidase activity in the absence of cells (Figure 4B.).
Figure 4. TQ solvents do not induce sialidase activity in BMC-2 cells and TQ does not exhibit sialidase activity in the absence of cells

A) BMC-2 cells were incubated overnight on 12mm circular glass slides in DMEM medium containing 5% fetal calf serum at 37°C. After removal of medium, 0.2mM 4-MUNANA substrate in TBS was added with DAKO mounting medium to cells alone (control), with 11.2mg/ml killed *Mycobacterium butyricum* (MYCO), dimethyl sulfoxide (DMSO), or 57% DMSO in TBS pH7.4. The free 4-methylumbelliferone fluorescence was measured at 450nm (blue color) following excitation at 365nm. Fluorescent images were acquired at one minute intervals using epi-fluorescent microscopy (40X objective). This experiment was repeated 3 times resulting in similar results.

B) Sialidase specific substrate 4-MUNANA was added with DAKO mounting medium alone (control), with 1mg/ml TQ, 100μg/ml TQ or Neuraminidase (*C. Perfringens*) to a glass slide. The free 4-methylumbelliferone fluorescence was measured at 450nm (blue color) following excitation at 365nm. Fluorescent images were acquired at one minute intervals using epi-fluorescent microscopy (40X objective). These data represent one of four individual experiments.
### A

<table>
<thead>
<tr>
<th>Phase</th>
<th>Control</th>
<th>MYCO</th>
<th>TQ in 57% DMSO in TBS</th>
<th>DMSO in TBS</th>
<th>57% DMSO in TBS</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td><img src="image1.png" alt="Image" /></td>
<td><img src="image2.png" alt="Image" /></td>
<td><img src="image3.png" alt="Image" /></td>
<td><img src="image4.png" alt="Image" /></td>
<td><img src="image5.png" alt="Image" /></td>
</tr>
<tr>
<td>1 min</td>
<td><img src="image6.png" alt="Image" /></td>
<td><img src="image7.png" alt="Image" /></td>
<td><img src="image8.png" alt="Image" /></td>
<td><img src="image9.png" alt="Image" /></td>
<td><img src="image10.png" alt="Image" /></td>
</tr>
<tr>
<td>2 min</td>
<td><img src="image11.png" alt="Image" /></td>
<td><img src="image12.png" alt="Image" /></td>
<td><img src="image13.png" alt="Image" /></td>
<td><img src="image14.png" alt="Image" /></td>
<td><img src="image15.png" alt="Image" /></td>
</tr>
<tr>
<td>3 min</td>
<td><img src="image16.png" alt="Image" /></td>
<td><img src="image17.png" alt="Image" /></td>
<td><img src="image18.png" alt="Image" /></td>
<td><img src="image19.png" alt="Image" /></td>
<td><img src="image20.png" alt="Image" /></td>
</tr>
</tbody>
</table>

### B

<table>
<thead>
<tr>
<th>Control</th>
<th>Neuraminidase</th>
<th>TQ</th>
</tr>
</thead>
<tbody>
<tr>
<td><img src="image21.png" alt="Image" /></td>
<td><img src="image22.png" alt="Image" /></td>
<td><img src="image23.png" alt="Image" /></td>
</tr>
</tbody>
</table>
4.2 TQ preferentially activates Neu4

Previous research in our lab determined that Neu1 was involved in ligand-induced TLR4 activation through the removal of sialic acids from glycosylated residues on the receptor [16]. Since there are four known forms of mammalian sialidase, the following experiments were performed to determine which sialidase is activated upon treatment with TQ.

4.2.1 Anti-Neu antibodies were able to bind to non-permeabilized and permeabilized mouse macrophage cells

The only anti-neuraminidase antibodies available at the time of these experiments were neutralizing antibodies against the four human forms of Neu sialidase. To determine if the antibodies were able to bind to mouse cells expressing Neu sialidases, immunocytochemistry techniques were employed using antibodies against the four Neu sialidases with fixed permeabilized and non-permeabilized BMC-2 mouse cell line and primary mouse bone marrow derived macrophage cells. Specific Alexa Fluor 488 labeled secondary antibodies were used to detect primary anti-Neu antibodies and viewed using epi-fluorescent microscopy. Controls using only the secondary antibody to treat cells were obtained to ensure secondary antibody was specifically binding the primary antibody. Neu1 and Neu4 were found to be expressed in permeabilized and non-permeabilized BMC-2 and primary mouse macrophage cells. Neu2 was detected in permeabilized and non-permeabilized primary mouse macrophage cells but only in non-permeabilized BMC-2 cells (Figure 5. and Figure 6.). Neu3 was not detected in these cells, perhaps due to the antibody specificity for human Neu3.
Figure 5. Identification of membrane-associated Neu sialidase in BMC-2 macrophage cells

BMC-2 cells were incubated on 12 mm circular glass slides in medium containing 5% fetal calf serum for 24 hours at 37°C. After removal of medium, cells were fixed with 4% paraformaldehyde and left in TBS or permeabilized with 0.2% Triton-X in TBS. Cells were blocked using 5% bovine serum albumin in 1x Tris buffered saline with 0.1% Tween. Cells were then left in TBS (control- No primary antibody) or treated with one of 1μg/ml rabbit anti-human anti-Neu1 antibody, 1μg/ml mouse anti-human anti-Neu2 antibody, 1μg/ml mouse anti-human anti-Neu3 antibody, or 1μg/ml rabbit anti-human anti-Neu4 antibody for 60 minutes at 37°C. Cells were washed and treated with either goat anti-mouse AlexaFluor 488 or goat anti-rabbit AlexaFluor 488 at a 1:700 dilution for 60 minutes at 37°C. Fluorescent images were taken using epi-fluorescent microscopy (40X objective). This data represents two individual experiments.
BMC-2 Cells

Control | αNeu1 | αNeu2 | αNeu3 | αNeu4
---|---|---|---|---

Non-Permeablized

αNeu1 | αNeu2 | αNeu3 | αNeu4

Permeablized

αNeu4
Figure 6. Identification of membrane-associated Neu sialidase in wild type mouse primary macrophage cells

Wild type mouse bone marrow derived primary macrophages were grown on 12 mm circular glass slides in RPMI 1640 medium containing 10% fetal calf serum, 20% monocyte colony stimulating factor (M-CSF) and 1x L-glutamine-penicillin-streptomycin (Pen-Strep) for 8 days at 37°C. After removal of medium, cells were fixed with 4% paraformaldehyde and left in TBS or permeabilized with 0.2% Triton-X in TBS. Cells were blocked with 5% bovine serum albumin in 1x Tris buffered saline with 0.1% Tween. Cells were then left in TBS (control – No primary antibody) or treated with one of 1μg/ml rabbit anti-human anti-Neu1 antibody, 1μg/ml mouse anti-human anti-Neu2 antibody, 1μg/ml mouse anti-human anti-Neu3 antibody, or 1μg/ml rabbit anti-human anti-Neu4 antibody for 60 minutes at 37°C. Cells were washed and treated with either goat anti-mouse AlexaFluor 594 or goat anti-rabbit AlexaFluor 594 at a dilution of 1:700 for 60 minutes at 37°C. Fluorescent images were taken using epi-fluorescent microscopy (40X objective). This figure is representative of two individual experiments.
Primary Bone Marrow Derived Mouse Macrophage Cells

Control    αNeu1    αNeu2    αNeu3    αNeu4

Non-Permeablized

Control    αNeu1    αNeu2    αNeu3    αNeu4

Permeablized

αNeu4

Non-Permeablized    Permeablized
4.2.2 Anti-Neu4 antibody completely inhibited TQ-induced sialidase activation in mouse BMC-2 macrophage cells and human HEK-TLR4 cells

Live cell sialidase assays were performed using neutralizing antibodies against Neu1-4 to determine which sialidase was activated with TQ treatment. These assays were performed with BMC-2 cells as well as HEK-TLR4 cells because the human anti-Neu3 antibody poorly binds to mouse Neu3. In the BMC-2 cells, treatment which combined the antibodies against all four Neu sialidase was able to inhibit TQ-induced sialidase activity. Anti-Neu4 was the only antibody able to completely inhibit TQ-induced sialidase activity in these cells. Treatment with anti-Neu1 antibody caused partial inhibition of TQ-induced sialidase activity whereas anti-Neu2 antibody was unable to significantly inhibit the sialidase activity (Figure 7.). The antibody and TQ treatments were added to the live mouse macrophage cells at the same time, which may have resulted in a slight decrease in blue fluorescence due to the decreased concentration of TQ with the added volume of the antibody. Although the anti-Neu3 antibody used was unable to bind mouse Neu3, there was a slight reduction in emitted fluorescence. In HEK-TLR4 cells, anti-Neu1 and anti-Neu2 antibodies did not inhibit TQ-induced sialidase activation (Figure 8.). Once again only treatment with the anti-Neu4 antibody was able to completely inhibit TQ-induced sialidase activity. The addition of anti-Neu1 and anti-Neu3 antibodies as well as DANA, an inhibitor of Neu2 and Neu3, to TQ-treated BMC-2 cells did not cause an inhibition of the TQ-induced sialidase activity (Figure 9.). Overall the anti-Neu4 was able to completely inhibit TQ-induced sialidase activity whereas; antibodies against Neu1, 2, and 3 did not fully inhibit this activation.
BMC-2 cells were incubated on 12 mm circular glass slides in DMEM medium containing 5% fetal calf serum for 24 hours at 37°C. After removal of medium, 0.2 mM 4-MUNANA (4-MU) substrate [2’-(4-methyllumebelliferyl)-α-D-N-acetylneuraminic acid] (Sigma) in Tris buffered saline pH 7.4 was added with mounting medium (Dako) to cells alone (Control), with 333 μg/ml lipopolysaccharide (LPS), 333 μg/ml thymoquinone (TQ), or 250 μg/ml TQ and 25 μg/ml rabbit anti-human anti-Neu1 antibody, 25 μg/ml mouse anti-human anti-Neu2 antibody, 25 μg/ml mouse anti-human anti-Neu3 antibody, or 25 μg/ml rabbit anti-human anti-Neu4 antibody. One glass slide with cells was pre-treated with anti-Neu4 antibody for 5 minutes. The substrate was hydrolyzed by activated sialidase to give free 4-methylumbelliferone which has a fluorescence emission at 450 nm (blue color) following excitation at 365 nm. Fluorescent images were taken at one minute time intervals using epi-fluorescent microscopy (40X objective). Fluorescence in pictures was analyzed by averaging the red, green and blue channels of 50 points proximal to cells using ImageJ software. Mean fluorescence was graphed using GraphPad Prism software. Significance was measured by one way ANOVA tests and Bonferroni’s multiple comparison tests at 95% confidence with P values < 0.0001. Differences between anti-Neu1 and anti-Neu3 treatment to control as well as anti-Neu4 and pre-treatment with anti-Neu4 to cells treated with TQ and LPS were significant though not shown in figure. These data represent one of three individual experiments.
Figure 8. Identification of membrane-associated Neu sialidase induced by TQ treatment in HEK-TLR4 cells

Human embryonic kidney epithelial cells stably transfected with human TLR4 (HEK-TLR4) were incubated for 24 hours on 12mm circular glass slides in DMEM medium containing 5% fetal calf serum and 0.8μg/ml G418 at 37°C. After removal of medium, 0.2mM 4-MUNANA (4-MU) substrate in Tris buffered saline pH 7.4 was added with mounting medium (Dako) to cells alone (Control), 333μg/ml lipopolysaccharide (LPS), 333μg/ml thymoquinone (TQ), or 250μg/ml TQ and 25μg/ml rabbit anti-human anti-Neu1 antibody, 25μg/ml mouse anti-human anti-Neu2 antibody, 25μg/ml mouse anti-human anti-Neu3 antibody, or 25μg/ml rabbit anti-human anti-Neu4 antibody. The substrate was hydrolyzed by activated sialidase to give free 4-methylumbelliferone which has a fluorescence emission at 450 nm (blue color) following excitation at 365 nm. Fluorescent images were taken at one minute time intervals using epi-fluorescent microscopy (40X objective). Fluorescence in pictures was analyzed by averaging the red, green and blue channels of 50 points proximal to cells using ImageJ software. Mean fluorescence was graphed using GraphPad Prism software. P values represent significant differences at 95% confidence using the Bonferroni’s multiple comparison tests to compare all treatments. This figure represents two individual experiments.
Figure 9. Identification of Neu sialidase induced by TQ treatment of BMC-2 cells with Anti-Neu antibodies and DANA

BMC-2 cells were incubated on 12 mm circular glass slides in medium containing 5% fetal calf serum for 24 hours at 37°C. After removal of medium, 0.2mM 4-MUNANA (4-MU) substrate in Tris buffered saline pH 7.4 was added with mounting medium (Dako) to cells alone (Control), with 333μg/ml thymoquinone (TQ), or 250μg/ml TQ and 250μg/ml Tamiflu, 50μg/ml rabbit anti-human anti-Neu1 antibody, 250μg/ml mouse anti-human anti-Neu3 antibody, DANA, or a combination of antibodies and DANA. The free 4-methylumbelliferone fluorescence was measured at 450nm (blue color) following excitation at 365nm. Fluorescent images were taken at one minute time intervals using epi-fluorescent microscopy (40X objective). Significance was assessed using one way AVONA tests and Beriferroni’s multiple comparison at 95% confidence with a P value of <0.0001. This figure is representative of three individual experiments.
4.2.3 Sialidase activity was measured in primary bone marrow derived macrophages isolated from Neu4 knock-out mice to confirm Neu4 activation by TQ treatment

In order to confirm that it was Neu4 sialidase activity induced by TQ, wild type (WT), Neu1 deficient (NeuIn), and Neu4KO primary mouse bone marrow derived macrophage cells were used in live cell sialidase assays. NeuIn primary macrophage cells had a hypomorphic cathepsin A that had a secondary reduction of the Neu1 activity. The fluorescent pictures acquired on the epi-fluorescent microscope were analyzed by averaging the red, green and blue channels of 50 points per picture surrounding the cells and performing one way ANOVA tests to determine significance. TQ was able to induce sialidase activity in WT and NeuIn macrophage cells (Figure 10.) but treatment with TQ did not produce a significant sialidase activity in Neu4KO macrophage cells (Figure 11.). These results support the hypothesis that Neu4 is the sialidase enzyme activated by TQ treatment.
Figure 10. TQ-induced sialidase activity in wild type mouse primary macrophage cells and Neu1 deficient human fibroblast cells

A) Bone marrow derived primary macrophage cells isolated from wild type mice were grown on 12 mm circular glass slides in DMEM medium containing 10% fetal calf serum, 20% monocyte colony stimulating factor (M-CSF) and 1x L-glutamine-penicillin-streptomycin (Pen-Strep) for 9 days at 37°C. After removal of medium, 0.2mM 4-MUNANA (4-MU) substrate in Tris buffered saline pH 7.4 was added with mounting medium (Dako) to cells alone (Control), with 3.7mg/ml mycobacterium (MYCO), 333μg/ml thymoquinone (TQ), or 33μg/ml TQ. The substrate was hydrolyzed by activated sialidase to give free 4-methylumbelliferone which has a fluorescence emission at 450 nm (blue color) following excitation at 365 nm. Fluorescent images were taken at one minute time intervals using epi-fluorescent microscopy (40X objective). P values represent significant differences at 95% confidence using the Bonferroni’s multiple comparison tests to compare all treatments. These data are representative of three individual experiments.

B) 1140F01 Neu1 deficient human fibroblast cells were grown on 12mm circular glass slides in DMEM medium containing 5% horse serum and 3% fetal calf serum requiring 1x Trypsin-EDTA for 24 hours at 37°C in 5% carbon dioxide. After removal of medium, 0.2mM 4-MU was added with Dako mounting medium to cells alone (control), or with 333μg/ml TQ. The substrate was hydrolyzed by activated sialidase enzyme to give free 4-methylumbelliferone which has a fluorescence emission at 450nm (blue) following excitation at 365nm. Fluorescent images were taken at one minute intervals using epi-fluorescent microscopy (40X objective). P values represent significant differences at 95% confidence using the Bonferroni’s multiple comparison tests to compare all treatments. These data represent two individual experiments.
Figure 11. TQ treatment of live bone marrow derived primary macrophage cells isolated from Neu4 knockout mice does not induce sialidase activity

Bone marrow derived primary macrophage cells isolated from Neu4 knockout mice were grown on 12 mm circular glass slides in DMEM medium containing 10% fetal calf serum, 20% monocyte colony stimulating factor (M-CSF) and 1x L-glutamine-penicillin-streptomycin (Pen-Strep) for 9 days at 37°C. After removal of medium, 0.2mM 4-MUNANA (4-MU) substrate [2'- (4-methylumbelliferyl)-α-D-N-acetylneuraminic acid] (Sigma) in Tris buffered saline pH 7.4 was added with mounting medium (Dako) to cells alone (Control), with 333μg/ml lipopolysaccharide (LPS), 1.67mg/ml LPS, 3.7mg/ml mycobacterium (MYCO), 333μg/ml thymoquinone (TQ), or 33μg/ml TQ. The substrate was hydrolyzed by activated sialidase to give free 4-methylumbelliferone which has a fluorescence emission at 450 nm (blue color) following excitation at 365 nm. Fluorescent images were taken at one minute time intervals using epi-fluorescent microscopy (40X objective). Fluorescence in pictures was analyzed by averaging the red, green and blue channels of 50 points proximal to cells using ImageJ software. Mean fluorescence was graphed using GraphPad Prism software. P values represent significant differences at 95% confidence using the Bonferroni’s multiple comparison tests to compare all treatments. This figure represents three individual experiments.
4.3 TQ induces a pro-inflammatory response through sialidase Neu4

Previous data showed that the activation of Neu1 by LPS resulted in a signal cascade that caused subsequent activation and nuclear localization of NFκB as well as the production of pro-inflammatory cytokines [16]. In order to determine if TQ-induced Neu4 activity resulted in NFκB activation as well, the following experiments were performed to test for NFκB activation and pro-inflammatory cytokine production following treatment with TQ.

4.3.1 TQ treatment of macrophage cells stimulates NFκB activation and nuclear localization

To establish that TQ activates NFκB in macrophage cells, immunocytochemistry techniques were employed using antibodies against both NFκB p65 and IκBα. Due to the ability of the NFκB p65 antibody to bind both active and inactive forms of NFκB p65, a control was required to ensure that NFκB was being activated. Therefore, the IκBα antibody was used as a control because IκB is degraded upon activation of NFκB. NFκB activation was determined if there was an increase in NFκB-associated fluorescence and nuclear localization of the NFκB p65 signal or a decrease in the IκBα signal. TQ is able to activate NFκB in BMC2 cells at 5-15 minutes to a similar degree as LPS was able to at 45 minutes (Figure 12.). Images from Figure 12 and 13 were analyzed for NFκB activation using Corel PHOTO-PAINT 11 software. Treatments resulting in higher levels of fluorescence when incubated with anti-NFκB versus anti-IκBα antibodies were considered an activation of NFκB. When IκBα fluorescence was higher than NFκB fluorescence, there was no activation of NFκB. In HEK-TLR4 cells, TQ activates NFκB at 15 minutes to a similar degree as LPS at 60 minutes (Figure 13.). The HEK TLR4 cells are used throughout this research project as part of this project was to determine the effects of TQ treatment on TLR4 activation.
BMC-2 cells were grown on 12 mm circular glass slides in DMEM medium containing 5% fetal calf serum for 48 hours at 37°C. After removal of medium, cells were treated with 5µg/ml lipopolysaccharide (LPS) or 100µg/ml thymoquinone (TQ) for various lengths of time. Cells were fixed with 4% paraformaldehyde for 30 minutes and permeabilized with 0.2% Triton-X in TBS for 5 minutes. Control (no primary antibody) cells were treated with secondary antibody only. Cells were treated with 1:200 rabbit anti-NFκBp65 or 1:200 rabbit anti-IκB for 60 minutes at 37°C. Cells were washed and treated with goat anti-rabbit AlexaFluor 594 for 60 minutes at 37°C. Fluorescent images were taken using epi-fluorescent microscopy (40X objective). This figure represents one of ten individual experiments. Images were analyzed using Corel PHOTO-PAINT 11 software. Pictures were inverted and transformed to a grayscale 8-bit colour mode. Background was analyzed in four regions and averaged. Cells were circled using mask tool. Corrected pixel density for ten different cells in each image was assessed using the following formula: (Background-Mean) x pixels. Data was compiled using GraphPad Prism 5 software. Control in this analysis indicates the cells treated with primary and secondary antibodies but not with ligand.
Figure 13. TQ-induced NFκB activation in HEK-TLR4 cells

HEK-TLR4 cells were grown on 12 mm circular glass slides in DMEM medium containing 5% fetal calf serum and 0.8μg/ml G418 for 48 hours at 37°C. After removal of medium, cells were treated with 5μg/ml lipopolysaccharide (LPS) or 100μg/ml thymoquinone (TQ) for various lengths of time. Cells were fixed with 4% paraformaldehyde for 30 minutes and permeabilized with 0.2% Triton-X in TBS for 5 minutes. Control cells were treated with secondary antibody only. Cells were treated with 1:200 rabbit anti-NFκBp65 or 1:200 rabbit anti-IκB for 60 minutes at 37°C. Cells were washed and treated with goat anti-rabbit AlexaFluor 594 for 60 minutes at 37°C. Fluorescent images were taken using epi-fluorescent microscopy (40X objective). This figure represents one of five repeated experiments. Images were analyzed using Corel PHOTO-PAINT 11 software. Pictures were inverted and transformed to a grayscale 8-bit colour mode. Background was analyzed in four regions and averaged. Cells were circled using mask tool. Corrected pixel density for ten different cells in each image was assessed using the following formula: (Background-Mean) x pixels. Data was compiled using GraphPad Prism 5 software. Control in this analysis indicates the cells treated with primary and secondary antibodies but not with ligand.
To confirm these results western blot analyses were performed on nuclear extracts from HEK-TLR4 cells and primary mouse macrophage cell lysates that were treated with TQ or left untreated as controls. The blots were probed with phospho-specific NFκB p65 antibody. Optimal activation of NFκB p65 subunit requires phosphorylation in the transactivation domain that interacts with the inhibitor IκB. The largest amount of phosphorylated NFκB was found in the nuclear extracts of cells stimulated with TQ for 10 minutes (Figure 14C). The primary macrophage cell lysates confirmed this increase in phosphorylated NFκB after treatment with TQ (Figure 14D.). Electrophoretic mobility shift assay (EMSA) techniques were also employed on BMC-2 (data not shown) and HEK-TLR4 nuclear extracts incubated with biotinylated NFκB oligonucleotide probe to confirm the nuclear localization and DNA binding of TQ-activated NFκB. Blots were incubated with streptavidin-HRP and analyzed with Pierce Lightshift EMSA kit chemiluminescence and X-ray film. Although there is evidence of increased band density after 5-10 minutes of treatment with TQ, the results of the EMSA experiments were less definitive than the results from the Western blot and NFκB immunocytochemistry analyses due to high amounts of NFκB signal in the control samples (Figure 14A and B, BMC-2 data not shown). An unlabeled cold competitor oligonucleotide probe was able to reduce the TQ-induced NFκB band at 250ng and complete abolish the band at 500ng (Figure 14B.).

4.3.2 Neuraminidase inhibitor Tamiflu inhibits TQ-induced NFκB activation

To establish that TQ-induced NFκB activation was the result of TQ-induced sialidase activity the previous immunocytochemistry techniques were repeated on BMC-2 mouse macrophage cells pre-treated with Tamiflu. Tamiflu was able to inhibit the NFκB activation in BMC-2 macrophages cells following treatment with TQ and LPS (Figure 15A.). Nuclear localization of NFκB in the TQ and LPS treated cells can be viewed after the conversion of
Figure 14. Nuclear localization and DNA binding of TQ-induced NFκB p65 activation in HEK-TLR4 cells and wild type mouse primary macrophage cells determined with EMSA and Western immunoblots

A) HEK-TLR4 cells were grown in DMEM medium containing 5% fetal calf serum and 0.8μg/ml G418 at 37°C. HEK-TLR4 cells were treated with 100μg/ml TQ for different times from 1 minute to 30 minutes. Nuclear extraction of the cells was performed using the Pierce nuclear extraction kit. Protein in extracts was quantified using the Bradford reagent method. Extracts were treated with 5ng of labeled NFκB probe and Pierce Lightshift chemiluminescent electrophoretic mobility shift assay (EMSA) kit contents according to protocol for 30 minutes. Extracts were run on an 8% acrylamide gel at 70V. Transferred to nylon membrane (Millipore) at 100mA for 30 minutes. Results were detected using chemiluminescent substrate from EMSA kit and photo film. This figure represents one of fifteen individual experiments.

B) HEK-TLR4 cells were grown in DMEM medium containing 5% fetal calf serum and 0.8μg/ml G418 at 37°C. HEK-TLR4 cells were treated with 100μg/ml TQ for different times from 1 minute to 30 minutes. Nuclear extraction of the cells was performed using Pierce nuclear extraction kit. Extracts were quantified using Bradford reagent method. Extracts were treated with 5ng of labeled NFκB probe, various concentrations of unlabeled NFκB probe (cold competitor, CC) and Pierce Lightshift chemiluminescent EMSA kit contents according to protocol for 30 minutes. Extracts were run on an 8% acrylamide gel at 70V. Transferred to nylon membrane (Millipore) at 100mA for 30 minutes. Results were detected using chemiluminescent substrate from EMSA kit and photo film. This figure represents one of four repeated experiments.

C) HEK-TLR4 cells were grown in DMEM medium containing 5% fetal calf serum and 0.8μg/ml G418 at 37°C. HEK-TLR4 cells were treated with 100μg/ml TQ for various times from 1 to 30 minutes. Nuclear extraction of the cells was performed using Pierce nuclear extraction kit. Extracts were quantified using Bradford reagent method. Nuclear extracts containing 20μg of protein were boiled with sample and lysis buffers. Samples were run on an 8% acrylamide SDS-PAGE gel. Gel was transferred to a PVDF membrane and membrane was probed with 1:4000 phospho-specific NFκBp65 antibody. Results were detected using chemiluminescent substrate and x-ray film. Loading controls were performed using 1:35000 β-Actin antibody. These data represent one of three repeated experiments.

D) Cell lysates were obtained from primary macrophage cells derived from wild type mice after being left untreated (control), or treated with 100μg/ml TQ. Lysates containing 20μg of protein were boiled with sample and lysis buffers. Samples were run on an 8% acrylamide SDS-PAGE gel. Gel was transferred to a PVDF membrane and membrane was probed with 1:4000 phospho-specific NFκBp65 antibody. Results were detected using chemiluminescent substrate and x-ray film. These data represent one of two repeated experiments. Loading controls were performed using 1:35000 β-Actin antibody.
A

**Treatment Time in min**

<table>
<thead>
<tr>
<th></th>
<th>TQ 30</th>
<th>TQ 15</th>
<th>TQ 10</th>
<th>TQ 5</th>
<th>TQ 1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ctrl</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>CC 5ng</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>CC 250ng</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>CC 500ng</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>αNFkB Ab</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

**HEK TLR4 Nuclear Extracts TQ Time Course**

B

C

**Wild Type Primary Macrophage Cell Lysates**

![](image1)

![](image2)

![](image3)
Figure 15. Tamiflu blocks TQ-induced NFκB activation in BMC-2 macrophage cells

A) BMC-2 cells were grown on 12 mm circular glass slides in DMEM medium containing 5% fetal calf serum for 48 hours at 37°C. After removal of medium, cells were left untreated or treated with 200μM Tamiflu for 30 minutes at 37°C. Inhibitors were removed and cells were treated with 5ug/ml lipopolysaccharide (LPS) or 100ug/ml thymoquinone (TQ) for 30 minutes at 37°C. Cells were fixed with 4% paraformaldehyde for 30 minutes and permeabilized with 0.2% Triton-X in TBS for 5 minutes. Cells were treated with 1:200 rabbit anti-NFκBp65 or 1:200 rabbit anti-IκB for 60 minutes at 37°C. Cells were washed and treated with goat anti-rabbit AlexaFluor 594 for 60 minutes at 37°C. Fluorescent images were taken using epi-fluorescent microscopy (40X objective). These data are representative of four individual experiments.

B) Pictures were converted to grey scale and inverted using Adobe Photoshop CS2 to emphasize the nuclear localization of NFκB after treatment with LPS and TQ.

C) Cell lysates were obtained from HEK TLR4 cells. Lysates containing 20μg of protein were boiled with sample and lysis buffers. Samples were run on an 8% acrylamide SDS-PAGE gel. Gel was transferred to a PVDF membrane and membrane was probed with 1:1000 phosho-specific NFκBp65 (RelA) antibody. Results were detected using chemiluminescent substrate and x-ray film. Loading controls were performed using 1:1000 GAPDH antibody. These data are representative of two individual experiments.
pictures acquired on the epi-fluorescent microscope into 8-bit inverted pictures using Image J software (Figure 15B.).

To confirm these results western blot analysis was done with cell lysates from HEK-TLR4 cells pre-treated with Tamiflu for 30 minutes followed by stimulation with TQ for 15 minutes. The blots were probed using phospho-specific NFκBp65 antibody. Tamiflu inhibited the TQ-induced NFκB activation in HEK-TLR4 cells (Figure 15C.).

4.3.3 TQ treatment in mice up regulates pro-inflammatory cytokine production in vivo

Cytokine analyses were performed to determine if mice treated with TQ were able to induce pro-inflammatory responses. Cytokine profile arrays of 40 different cytokines and chemokines were executed on the serum extracted from normal (wild type), Neu1 deficient (NeuIn) and Neu4 knock-out (Neu4KO) mice before and 5 hours after an intraperitoneal injection of 2mg/kg of TQ. Serum was incubated with pre-labeled membranes containing the antibodies for 40 different cytokines. The wild type and NeuIn mice had a general up-regulation of cytokines after treatment with TQ whereas the Neu4KO mice levels of cytokine production remained the same or decreased after treatment with TQ (Figure 16A-C). Several pro-inflammatory cytokines were expressed in the serum of the wild type and NeuIn mice after treatment with TQ including sICAM-1, IFN-γ, IL-1α, β and ra, IL-6, TREM-1 and TNF-α (Figure 17.). The largest increases were in the production of sICAM-1, a 13.8 fold increase in wild type mice, and IFN-γ, IL-1α, B and ra, IL-6, and TREM-1, which all had over 3 fold increases in production after TQ treatment in wild type and NeuIn mice. Chemokines such as CXCL1, CCL2, MIP-1α and β (CCL 3 and 4 respectively), and MIP-2 (CXCL2) were also up regulated by 2 fold or more after the TQ treatment of wild type and NeuIn mice (Figure 18). Wild type and NeuIn mice treated with TQ
Figure 16. Cytokine array membranes used for the analysis of TQ-induced cytokine production in serum from wild type, Neu1 deficient (NeuIn), and Neu4 knock-out (Neu4KO) mice

Blood was collected from wild type mice before (control) and 5 hours after intraperitoneal treatment with 2mg/kg thymoquinone. Serum from blood was used for cytokine analysis with R&D System Array Profiling kit. The serum was mixed with the antibody detection cocktail for 1 hour at room temperature followed by an overnight incubation with the membranes overnight at 4°C. Membranes were then incubated with streptavidin-HRP for 30 minutes at room temperature. Cytokines on membranes were detected and analyzed using Pierce chemiluminescent detection reagents and Fluorchem HD2 Imaging System.

A) Membrane treated with serum collected from wild type mice before and after treatment with TQ. These figures represent one of four repeated experiments

B) Membrane treated with serum collected from NeuIn mice before and after treatment with TQ.

C) Membrane treated with serum collected from Neu4KO mice before and after treatment with TQ. These figures represent one of two repeated experiments.
demonstrated no increase in the anti-inflammatory cytokine IL-10 and little increase in other anti-inflammatory cytokines such as IL-4 with a fold increase between 1.5 and 2 (Figure 19).

4.3.4 TQ-induced expression of pro-inflammatory cytokines is dependent on Neu4 sialidase

The role of Neu4 sialidase in TQ-induced cytokine production was evaluated using the serum extracted from wild type, Neu1 deficient (NeuIn) and Neu4KO mice before and 5 hours after treatment with TQ. Cytokine profile arrays of 40 cytokine and chemokines were performed. Pro-inflammatory cytokine and chemokine up regulation with TQ treatment was evident in both wild type and NeuIn mice (Figure 16-19). In the Neu4KO mice, there was a decrease in pro-inflammatory cytokine production after treatment with TQ (Figure 17). The production of GM-CSF, I-309, sICAM-1, IFN-γ, IL-1β, IL-3, IL-7, M-CSF, TNF-α and TREM-1 in Neu4KO mice after treatment with TQ was reduced by half or more. There was an overall reduction in cytokine and chemokine production after TQ treatment in the Neu4KO mice which was a large difference from the wild type and NeuIn mice treated with TQ (Figure 17-19). It should be noted that the wild type and NeuIn mice became lethargic after receiving TQ treatment whereas the Neu4KO mice appeared to have relatively normal behavior. This difference may be due to the extensive inflammatory response occurring in the animals with activated Neu4.

4.3.5 TQ up regulates key pro-inflammatory cytokine genes

To confirm the up regulation of pro-inflammatory cytokines, polymerase chain reaction (PCR) was performed on RNA extracts from untreated HEK-TLR4 cells as well as cells treated with TQ for 2, 4, 6 or 8 hours and cells treated with LPS for 4 hours. The treatment with LPS was intended for use as a positive control. The cytokines tested were chosen based on availability of primer for these studies and cytokines which are known for their prominent pro-inflammatory response. There was an increase in the signal for the production of IL-6, TNF-α and MIP-1β in
Figure 17. Analysis of TQ-induced pro-inflammatory cytokine production in serum from wild type, Neu1 deficient and Neu4 knockout mice

Blood was collected from wild type, Neu1 deficient (NeuIn) and Neu4 knock-out (Neu4KO) mice before (control) and 5 hours after intraperitoneal treatment with 2mg/kg thymoquinone. Serum from blood was used for cytokine analysis with R&D System Array Profiling kit. The serum was mixed with the antibody detection cocktail for 1 hour at room temperature followed by an overnight incubation with the membranes overnight at 4°C. Membranes were then incubated with streptavidin-HRP for 30 minutes at room temperature. Cytokines on membranes were detected and analyzed using Pierce chemiluminescent detection reagents and Fluorchem HD2 Imaging System. Membrane fluorescence was analyzed as pixel density correct to the background using the Fluorchem HD2 Imaging system with associated computer software. Fold increase was determined by dividing the pixel density of the control by the pixel density after treatment with TQ. Fold increases of greater than one represent an increase in cytokine production after treatment with TQ whereas fold values less than one represent a decrease in cytokine production after treatment. Data was assembled using GraphPad Prism 5 software. These data represent three of eight individual experiments.
Figure 18. Analysis of TQ-induced pro-inflammatory chemokine production in serum from wild type, Neu1 deficient and Neu4 knockout mice

Blood was collected from wild type, Neu1 deficient (NeuIn) and Neu4 knock-out (Neu4KO) mice before (control) and 5 hours after intraperitoneal treatment with 2mg/kg thymoquinone. Serum from blood was used for cytokine analysis with R&D System Array Profiling kit. The serum was mixed with the antibody detection cocktail for 1 hour at room temperature followed by an overnight incubation with the membranes overnight at 4°C. Membranes were then incubated with streptavidin-HRP for 30 minutes at room temperature. Cytokines on membranes were detected and analyzed using Pierce chemiluminescent detection reagents and Fluorchem HD2 Imaging System. Membrane fluorescence was analyzed as pixel density correct to the background using the Fluorchem HD2 Imaging system with associated computer software. Fold increase was determined by dividing the pixel density of the control by the pixel density after treatment with TQ. Fold increases of greater than one represent an increase in cytokine production after treatment with TQ whereas fold values less than one represent a decrease in cytokine production after treatment. Data was assembled using GraphPad Prism 5 software. These data represent three of eight individual experiments.
Figure 19. Analysis of TQ-induced production of other cytokines in serum from wild type, Neu1 deficient and Neu4 knockout mice

Blood was collected from wild type, Neu1 deficient (NeuIn) and Neu4 knock-out (Neu4KO) mice before (control) and 5 hours after intraperitoneal treatment with 2mg/kg thymoquinone. Serum from blood was used for cytokine analysis with R&D System Array Profiling kit. The serum was mixed with the antibody detection cocktail for 1 hour at room temperature followed by an overnight incubation with the membranes overnight at 4°C. Membranes were then incubated with streptavidin-HRP for 30 minutes at room temperature. Cytokines on membranes were detected and analyzed using Pierce chemiluminescent detection reagents and Fluorchem HD2 Imaging System. Membrane fluorescence was analyzed as pixel density correct to the background using the Fluorchem HD2 Imaging system with associated computer software. Fold increase was determined by dividing the pixel density of the control by the pixel density after treatment with TQ. Fold increases of greater than one represent an increase in cytokine production after treatment with TQ whereas fold values less than one represent a decrease in cytokine production after treatment. Data was assembled using GraphPad Prism 5 software. These data represent three of eight individual experiments.
the cells treated with TQ for 4 hours (Figure 20.). The RNA was treated with β-actin primer as a loading control. The general trend was that the more intense the band in the mRNA from cells treated with TQ for 4 hours, the higher the production of that cytokine seemed to be after treatment with TQ in vivo as seen in the cytokine arrays (Figure 16 to 19.).
Figure 20. TQ-induced increase in expression of mRNA of pro-inflammatory cytokines: IL-6, TNF-α and MIP-1β

HEK-TLR4 cells were left untreated (negative control) or treated with TQ for 2, 4, 6, or 8 hours, or with LPS for 4 hours as a positive control. RNA was extracted from HEK-TLR4 cells using the Ambion Tri reagent method. RNA then underwent reverse transcriptase polymerase chain reaction (PCR) to convert the RNA to cDNA. cDNA was then subjected to PCR with primers for B-actin (660bp, 40 cycles), IL-6 (295bp, 40 cycles), TNF-α (413bp, 30 cycles) and MIP-1β (295bp, 40 cycles). Samples were run on a 1.2% agarose gel containing Ethidium Bromide. Bands were visualized using Fluorchem HD2 Imaging System. These figures represent three repeated experiments for each cytokine.
4.4 TQ activates Neu4 sialidase through GPCR Gαᵢ subunit and MMP9

Unpublished data (Amith et al.) have shown that LPS-induced Neu1 sialidase activity was dependent on G protein coupled receptor (GPCR) G protein subunits and matrix metalloproteinase (MMP) [16]. Using LPS-induced Neu1 activation as a model, tests were performed to discover if GPCR and MMP were involved in TQ-induced Neu4 sialidase activity. Sialidase activity has also been associated with Trk tyrosine kinase receptors [9]. Therefore experiments were conducted to determine if TQ was acting through Trk tyrosine kinase receptor.

4.4.1 MMP and GPCR Gαᵢ subunit inhibitors are able to diminish TQ-induced sialidase activity

Inhibitors utilized in this study included broad range and specific inhibitors to MMPs, GPCR, GPCR-associated G proteins and TrkA tyrosine kinase receptors. Treatments with TQ and inhibitors, Pertussis toxin (PTX), galardin (GALA), Tamiflu (TAMI), Cholera toxin (CTX), Cholera toxin subunit B (CTXB), K252a, black seed oil (BSO) and suramin (SRMN), were used in a BMC-2 live cell sialidase assay where sialidase-associated fluorescence of hydrolyzed 4-MU was measured in the area surrounding the macrophage cells. Previous studies on sialidase activation influenced the choice of inhibitors used in this study to include inhibitors for sialidase, GPCR, MMP and TrkA. Of all the inhibitors tested, GPCR Gαᵢ inhibitor pertussis toxin (PTX), MMP inhibitor galardin (GALA) and sialidase inhibitor Tamiflu were able to inhibit the TQ-induced sialidase activity (Figure 21.). Other inhibitors including Suramin, an inhibitor that uncouples the G protein subunits from GPCR, and Cholera toxin (CTX), an inhibitor of the Gαₛ subunit, were not able to inhibit TQ-induced sialidase activity. Previous studies demonstrated that sialidase activation was evident after stimulation of TrkA tyrosine kinase receptors [9]. It was found that TQ was not acting through TrkA tyrosine kinase receptor to induce sialidase activity as
Figure 21. TQ-induced sialidase activity is inhibited with sialidase, GPCR Ga_i subunit and MMP inhibitors in BMC-2 macrophage cells

BMC-2 cells were incubated on 12 mm circular glass slides in medium containing 5% horse and 3% fetal calf serum for 5 hours at 37°C. After removal of medium, 0.2mM 4-MUNANA (4-MU) substrate [2’-(4-methylumbelliferyl)-α-D-N-acetylneuraminic acid] (Sigma) in Tris buffered saline pH 7.4 was added with mounting medium (Dako) to cells alone (Control), with 0.8mg/ml thymoquinone (TQ), 0.6mg/ml TQ and 25ng/ml Pertussis toxin (PTX), 500nM Galardin (GALA), 1mg/ml Tamiflu, 250μg/ml cholera toxin (CTX), 2.5mg/ml cholera toxin B (CTXB), 0.2μM K252a, 5%BSO in DMSO, or 100μM Suramin (SRMN). The free 4-methylumbelliferone fluorescence was measured at 450nm (blue color) following excitation at 365nm. Fluorescent images were taken at one minute time intervals using epi-fluorescent microscopy (40X objective). Fluorescence in pictures was analyzed by averaging the red, green and blue channels of 50 points proximal to cells using ImageJ software. Mean fluorescence was graphed using GraphPad Prism software. P values represent significant differences at 95% confidence using the Bonferroni’s multiple comparison tests to compare all treatments. These data represent one of six individual experiments.
K252a, an inhibitor of TrkA, was unable to inhibit the sialidase activity. The live cell sialidase assay was repeated with primary wild type these macrophage cells. Treatment of primary mouse macrophage cells with TQ and the Tamiflu, galardin and PTX inhibitors also resulted in inhibition of TQ-induced sialidase activity (Figure 2.).

4.4.2 MMP9 is involved in TQ-induced sialidase activity

Various inhibitors to MMPs were used to elucidate which MMP was involved in TQ-induced sialidase activity. TQ treatment was combined with various concentrations of galardin, which inhibits MMP1, 3, 8, and 9, and piperazine, which inhibits MMP1, 3, 7, and 9, in a live cell sialidase assay performed with BMC-2 macrophage cells. Piperazine was able to inhibit TQ-induced sialidase activity at concentrations as low as 2μg/ml (Figure 23.). Galardin was also able to inhibit the sialidase activity induced by TQ at concentrations from 1mg/ml to 5μg/ml (Figure 24A.). Both inhibitors selectively block the activity of MMPs 1, 3 and 9. Therefore, the MMP involved in TQ-induced sialidase activity could be either MMP1, 3, or 9. As MMP1 does not have elastase activity which we believe is important in sialidase activation [70], we focused on MMP3 and 9. Neutralizing antibodies to specific MMPs were used to determine the specific MMP involved in the TQ-induced sialidase activity. The live cell sialidase assay was conducted on BMC-2 cells using TQ treatment together with a neutralizing antibody against MMP9. The anti-MMP9 antibody was able to significantly inhibit TQ-induced sialidase activity (Figure 24B.). Significance was determined using a one way ANOVA test on the average of the RGB channels measured at 50 points surrounding the cells in the images acquired on the epi-fluorescent microscope.
Figure 22. TQ-induced sialidase activity is inhibited by sialidase, GPCR \(\text{G}_i\) subunit and MMP inhibitors in primary macrophage cells from wild type mice

Wild type mouse bone marrow derived primary macrophages were grown on 12 mm circular glass slides in RPMI 1640 medium containing 10% fetal calf serum, 20% monocyte colony stimulating factor (M-CSF) and 1x L-glutamine-penicillin-streptomycin (Pen-Strep) for 9 days at 37°C. After removal of medium, 0.2mM 4-MUNANA (4-MU) substrate in Tris buffered saline pH 7.4 was added with mounting medium (Dako) to cells alone (Control), with 333μg/ml lipopolysaccharide (LPS), 3.7mg/ml mycobacterium (MYCO), 333μg/ml thymoquinone (TQ), 33μg/ml TQ, 250μg/ml TQ and 250μg/ml Tamiflu, or 25μg/ml TQ and 500nM Galardin (GALA) or 25ng/ml Pertussis toxin (PTX). The substrate was hydrolyzed by activated sialidase to give free 4-methylumbelliferone which has a fluorescence emission at 450 nm (blue color) following excitation at 365 nm. Fluorescent images were taken at one minute time intervals using epi-fluorescent microscopy (40X objective). Fluorescence in pictures was analyzed by averaging the red, green and blue channels of 50 points proximal to cells using ImageJ software. Mean fluorescence was graphed using GraphPad Prism software. \(P\) values represent significant differences at 95% confidence using the Bonferroni’s multiple comparison tests to compare all treatments. These data are representative of two individual experiments.
<table>
<thead>
<tr>
<th>Treatment</th>
<th>Control</th>
<th>LPS</th>
<th>MYCO 333µg/ml</th>
<th>TQ 33µg/ml + Tamiflu</th>
<th>TQ 25µg/ml + Galardin</th>
<th>TQ 25µg/ml + PTX</th>
</tr>
</thead>
</table>

**Mean Fluorescence**

- TQ + PTX: P<0.0001
- TQ + Galardin: P<0.0001
- TQ + Tamiflu: P<0.0001
- TQ 33µg/ml: P<0.0001
- TQ 333µg/ml: P<0.0001
- MYCO: P<0.0001
- LPS: P<0.0001
- Ctrl: P<0.0001
Figure 23. TQ-induced sialidase activity in BMC-2 cells is inhibited by MMP inhibitor piperazine

BMC-2 cells were incubated on 12 mm circular glass slides in medium containing 5% fetal calf serum for 5 hours at 37°C. After removal of medium, 0.2mM 4-MUNANA (4-MU) substrate in Tris buffered saline pH 7.4 was added with mounting medium (Dako) to cells alone (Control), with 333μg/ml thymoquinone (TQ), or 250μg/ml TQ and various doses of piperazine (PIPZ). The substrate was hydrolyzed by activated sialidase to give free 4-methylumbelliferone which has a fluorescence emission at 450 nm (blue color) following excitation at 365 nm. Fluorescent images were taken at one minute time intervals using epi-fluorescent microscopy (40X objective). Fluorescence in pictures was analyzed by averaging the red, green and blue channels of 50 points proximal to cells using ImageJ software. Mean fluorescence was graphed using GraphPad Prism software.  $P$ values represent significant differences at 95% confidence using the Bonferroni’s multiple comparison tests to compare all treatments. These data represent one of three repeated experiments.
Figure 24. TQ-induced sialidase activity in BMC-2 cells is inhibited by the MMP inhibitor galardin and MMP9 neutralizing antibody

A) BMC-2 cells were incubated on 12 mm circular glass slides in medium containing 5% fetal calf serum for 5 hours at 37°C. After removal of medium, 0.2 mM 4-MUNANA (4-MU) substrate in Tris buffered saline pH 7.4 was added with mounting medium (Dako) to cells alone (Control), with 333 μg/ml thymoquinone (TQ), or 250 μg/ml TQ and 1 mg/ml Galardin or 500 nM Galardin (GALA). The substrate was hydrolyzed by activated sialidase to give free 4-methylumbelliferone which has a fluorescence emission at 450 nm (blue color) following excitation at 365 nm. Fluorescent images were taken at one minute time intervals using epi-fluorescent microscopy (40X objective). Fluorescence in pictures was analyzed by averaging the red, green and blue channels of 50 points proximal to cells using ImageJ software. Mean fluorescence was graphed using GraphPad Prism software. P values represent significant differences at 95% confidence using the Bonferroni’s multiple comparison tests to compare all treatments. These data represent one of ten individual experiments.

B) Previous procedure was repeated with BMC-2 cells. After removal of medium, cells were treated using 0.2 mM 4-MU and DAKO with cells alone (Control), 333 μg/ml thymoquinone (TQ), or 250 μg/ml TQ and 25 μg/ml rabbit anti-human anti-MMP9 antibody. The substrate was hydrolyzed by activated sialidase to give free 4-methylumbelliferone which has a fluorescence emission at 450 nm (blue color) following excitation at 365 nm. Fluorescent images were taken at one minute time intervals using epi-fluorescent microscopy (40X objective). Fluorescence in pictures was analyzed as above. P values represent significant differences at 95% confidence using the Bonferroni’s multiple comparison tests to compare all treatments. These figures represent one of three individual experiments.
To determine if the MMP9 was expressed on the cell surface, immunocytochemistry technique were employed with permeabilized and non-permeabilized cells in a similar manner as previously completed with the anti-Neu antibodies. The degree of fluorescence in each condition was examined. MMP9 was expressed on the cell surface of non-permeabilized BMC-2 cells but more so in permeabilized cells (Figure 25A.). These latter data with permeabilized cells were similar in the primary wild type mouse macrophage cells (Figure 25B.). A control with no primary antibody was completed in both experiments to ensure specific binding of the secondary antibody to the primary antibody.

4.4.3 Both GPCR $G_\alpha_i$ subunit and MMP9 are involved in TQ-induced NFκB activation

To elucidate the role of GPCR $G_\alpha_i$ subunit and MMP in TQ-induced cellular activation, the levels of NFκB activation were tested. The investigation of NFκB in this study was used to indicate a possible inflammatory response as NFκB acts as a transcription factor to induce transcription of cytokine genes. Immunocytochemistry techniques using antibodies against both NFκB p65 and IκBα were added to cells incubated with a broad range inhibitor for sialidase, an inhibitor to GPCR $G_\alpha_i$ subunit and broad range inhibitor for MMP. BMC-2 cells were pre-treated for 30 minutes with Tamiflu, galardin or PTX followed by a 30 minute stimulation with TQ. All three inhibitors were able to inhibit the TQ-induced NFκB activation (Figure 26A.).

To confirm these results, western blot analyses were completed on HEK-TLR4 nuclear extracts pre-treated with Tamiflu, galardin and PTX, for 30 minutes followed by a 30 minute stimulation with TQ. The membranes were immunoblotted using phospho-specific NFκBp65 antibody. Tamiflu fully blocked the TQ-induced NFκB activation whereas galardin and PTX only partially inhibited the activation of NFκB (Figure 26B.).
Figure 25. Detection of membrane associated MMP9 in BMC-2 macrophage cells and wild type mouse primary macrophage cells

A) BMC-2 cells were incubated on 12 mm circular glass slides in medium containing 5% fetal calf serum for 24 hours at 37°C. After removal of medium, cells were fixed with 4% paraformaldehyde and left in TBS or permeabilized with 0.2% Triton-X in TBS. Cells were then left in TBS (control with no primary antibody) or treated with 1μg/ml rabbit anti-human anti-MMP9 antibody for 60 minutes at 37°C. Cells were washed and treated with either goat anti-mouse AlexaFluor 488 or goat anti-rabbit AlexaFluor 488 for 60 minutes at 37°C. Fluorescent images were taken using epi-fluorescent microscopy (40X objective). These data represent one of two individual experiments.

B) Wild type mouse bone marrow derived primary macrophages were grown on 12 mm circular glass slides in RPMI 1640 medium containing 10% fetal calf serum, 20% monocyte colony stimulating factor (M-CSF) and 1x L-glutamine-penicillin-streptomycin (Pen-Strep) for 8 days at 37°C. After removal of medium, cells were fixed with 4% paraformaldehyde and left in TBS or permeabilized with 0.2% Triton-X in TBS. Cells were then left in TBS (control with no primary antibody) or treated with 1μg/ml rabbit anti-human anti-MMP9 antibody for 60 minutes at 37°C. Cells were washed and treated with either goat anti-mouse AlexaFluor 594 or goat anti-rabbit AlexaFluor 594 for 60 minutes at 37°C. Fluorescent images were taken using epi-fluorescent microscopy (40X objective). These data are representative of two individual experiments.
Figure 26. TQ-induced NFκB activation is reduced by sialidase, GPCR Gαi subunit, and MMP inhibitors

A) BMC-2 cells were grown on 12 mm circular glass slides in DMEM medium containing 5% fetal calf serum for 48 hours at 37°C. After removal of medium, cells were left untreated or treated with 400μM Tamiflu, 500nM Galardin (GALA), or 100ng/ml Pertussis toxin (PTX) for 30 minutes at 37°C. Inhibitors were removed and cells were treated with 1mg/ml thymoquinone (TQ) for 30 minutes at 37°C. Cells were fixed with 4% paraformaldehyde for 30 minutes and permeabilized with 0.2% Triton-X in TBS for 5 minutes. Cells were treated with 1:200 rabbit anti-NFκBp65 or 1:200 rabbit anti-IκB for 60 minutes at 37°C. Cells were washed and treated with goat anti-rabbit AlexaFluor 594 for 60 minutes at 37°C. Fluorescent images were taken using epi-fluorescent microscopy (40X objective). These data are representative of one of three individual experiments,

B) Nuclear extracts were obtained from HEK-TLR4 cells. Lysates containing 20μg of protein were boiled with sample and lysis buffers. Samples were run on an 8% acrylamide SDS-PAGE gel. Gel was transferred to a PVDF membrane and membrane was probed with 1:5000 phospho-specific NFκBp65 (RelA) antibody. Results were detected using chemiluminescent substrate and x-ray film. These data are representative of two experiments.
4.5 TQ trans-activates TLR4 through Neu4

Previous studies in the laboratory have shown that LPS binding to TLR4 receptors induces Neu1 sialidase activity. The LPS-induced Neu1 sialidase activity is required for the subsequent TLR4 activation in TLR-expressing cells [10]. It was proposed that TQ may also act in a similar mechanism to activate the TLR4 receptor.

4.5.1 TQ is not a TLR4 ligand

TQ treatment of live TLR-deficient HEK293 cells was able to induce sialidase activity in these cells in the absence of TLR4 (Appendix A Figure 38.). This observation suggests that TQ does not require TLR4 to induced sialidase activity in live cells.

4.5.2 TQ activates TLR4 in wild type and NeuIn mouse primary macrophage cells

Studies have shown that GPCR agonists can trans-activate EGFR independent of EGFR ligand [53]. This trans-activation of TLRs has not been established, although studies in the laboratory have shown that treatment with trans-sialidase derived from T. cruzi in macrophage cells causes the activation of TLR4 in the absence of LPS [16]. It was hypothesized that TQ may be able to trans-activate TLR4 independent of LPS. Given that TQ was not acting as a TLR4 ligand, experiments were conducted to determine if TQ-induced sialidase activity was able to activate TLR4. When TLR4 is activated by LPS, the receptor dimerizes and facilitates the formation of the TLR4/MyD88 complex for subsequent signal transduction. Immunocytochemistry co-localization of TLR4 and MyD88 was performed with wild type mouse macrophage cells. Alexa Fluor labeled secondary antibodies appearing green (λ488nm, TLR4) and red (λ594nm, MyD88) were used to visualize the localization of TLR4 and MyD88. The appearance of yellow colour indicated the co-localization of TLR4 with MyD88. TQ treatment
resulted in the co-localization of TLR4 with MyD88 suggesting that TQ was able to activate TLR4 in wild type primary macrophage cells (Figure 27A.).

Although Neu1 activation was not witnessed after TQ treatment, TLR4 activation upon treatment with LPS required Neu1 sialidase activity [10]. Therefore, TQ-induced TLR4 activation may also have been dependent on Neu1 sialidase activation. To determine if TQ treatment required Neu1 sialidase activity to activate TLR4, co-localization of TLR4 and MyD88 immunocytochemistry experiments were performed on Neu1 deficient (NeuIn) primary mouse macrophage cells. TQ treatment of NeuIn mouse macrophage cells resulted in TLR4 activation shown by the co-localization with MyD88 (Figure 27B.). These data may suggest that TQ-induced Neu4 sialidase activity may overcome the requirement of Neu1 sialidase for the activation of TLR4.

4.5.3 TQ is unable to activate TLR4 in Neu4 knock out macrophage cells

Co-localization of TLR4 and MyD88 immunocytochemistry experiments were carried out with primary Neu4KO mouse macrophage cells to determine if the TQ-induced Neu4 sialidase activity was important for the TLR4 activation following TQ treatment. TQ was unable to stimulate TLR4-MyD88 co-localization in Neu4KO cells suggesting that Neu4 is crucial for TQ-induced TLR4 activation (Figure 28.)
Figure 27. TQ-induced MyD88 co-localization with TLR4 in primary macrophage cells isolated from wild type and Neu1 deficient mice

A) Wild type mouse bone marrow derived primary macrophages were grown on 12 mm circular glass slides in DMEM medium containing 10% fetal calf serum, 20% monocyte colony stimulating factor (M-CSF) and 1x L-glutamine-penicillin-streptomycin (Pen-Strep) for 8 days at 37°C. After removal of medium, cells were treated with 5μg/ml lipopolysaccharide (LPS) or 100μg/ml thymoquinone (TQ) for 5 minutes. Cells were fixed with 4% paraformaldehyde for 30 minutes and permeabilized with 0.2% Triton-X in TBS for 5 minutes. Cells were treated with 1:200 rabbit anti-mouse anti-MyD88 and 1:200 rat anti-mouse anti-TLR4 for 60 minutes at 37°C. Cells were washed and treated with goat anti-rabbit AlexaFluor 594 for 60 minutes at 37°C then with rabbit anti-rat AlexaFluor 488 for 60 minutes at 37°C. Fluorescent images were taken using epi-fluorescent microscopy (40X objective) and confocal microscopy. Co-localization analysis was completed using ImageJ software. These data represent one of two individual experiments.

B) The same procedure was repeated with Neu1 deficient (NeuIn) mouse bone marrow derived primary macrophages. After removal of medium, cells were treated with 5μg/ml lipopolysaccharide (LPS) or 100μg/ml thymoquinone (TQ) for 5 minutes. Cells were treated with 1:200 rabbit anti-mouse anti-MyD88 and 1:200 rat anti-mouse anti-TLR4 for 60 minutes at 37°C. Cells were washed and treated with goat anti-rabbit AlexaFluor 594 for 60 minutes at 37°C then with rabbit anti-rat AlexaFluor 488 for 60 minutes at 37°C. Fluorescent images were taken using epi-fluorescent microscopy (40X objective) and confocal microscopy. Co-localization analysis was completed using ImageJ software. These data are representative of two individual experiments.
Figure 28. Lack of TQ-induced MyD88 co-localization with TLR4 in primary macrophages cells isolated from Neu4 knockout mice

Neu4 knock out mouse bone marrow derived primary macrophages were grown on 12 mm circular glass slides in DMEM medium containing 10% fetal calf serum, 20% monocyte colony stimulating factor (M-CSF) and 1x L-glutamine-penicillin-streptomycin (Pen-Strep) for 8 days at 37°C. After removal of medium, cells were treated with 5μg/ml lipopolysaccharide (LPS) or 100μg/ml thymoquinone (TQ) for 5 minutes. Cells were fixed with 4% paraformaldehyde for 30 minutes and permeabilized with 0.2% Triton-X in TBS for 5 minutes. Cells were treated with 1:200 rabbit anti-mouse anti-MyD88 and 1:200 rat anti-mouse anti-TLR4 for 60 minutes at 37°C. Cells were washed and treated with goat anti-rabbit AlexaFluor 594 for 60 minutes at 37°C then with rabbit anti-rat AlexaFluor 488 for 60 minutes at 37°C. Fluorescent images were taken using epi-fluorescent microscopy 40X objective and 100X oil emersion objective. Co-localization analysis was completed using ImageJ software. These data are representative of two experiments.
<table>
<thead>
<tr>
<th></th>
<th>TLR4</th>
<th>MYD88</th>
<th>Overlay</th>
<th>Percent Overlay</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td><img src="image1" alt="Control TLR4" /></td>
<td><img src="image2" alt="Control MYD88" /></td>
<td><img src="image3" alt="Control Overlay" /></td>
<td>0%</td>
</tr>
<tr>
<td>LPS</td>
<td><img src="image4" alt="LPS TLR4" /></td>
<td><img src="image5" alt="LPS MYD88" /></td>
<td><img src="image6" alt="LPS Overlay" /></td>
<td>31%</td>
</tr>
<tr>
<td>MYCO</td>
<td><img src="image7" alt="MYCO TLR4" /></td>
<td><img src="image8" alt="MYCO MYD88" /></td>
<td><img src="image9" alt="MYCO Overlay" /></td>
<td>35%</td>
</tr>
<tr>
<td>TQ 100µg/ml</td>
<td><img src="image10" alt="TQ TLR4" /></td>
<td><img src="image11" alt="TQ MYD88" /></td>
<td><img src="image12" alt="TQ Overlay" /></td>
<td>0%</td>
</tr>
</tbody>
</table>
4.5.4 GPCR $G_{\alpha_i}$ subunit and MMP9 are important for TQ-induced TLR4 activation

Further TLR4-MyD88 co-localization experiments were performed to confirm that GPCR $G_{\alpha_i}$ and MMP were involved in TQ-induced TLR4 activation. Primary wild type mouse macrophage cells were pre-treated with Tamiflu, galardin or PTX for 30 minutes followed by TQ stimulation for 5 minutes. All three inhibitors were able to inhibit the co-localization of TLR4 and MyD88 that was seen in the cells with TQ stimulation only (Figure 29.).
Wild type mouse bone marrow derived primary macrophages were grown on 12 mm circular glass slides in DMEM medium containing 10% fetal calf serum, 20% monocyte colony stimulating factor (M-CSF) and 1x L-glutamine-penicillin-streptomycin (Pen-Strep) for 8 days at 37°C. After removal of medium, cells were treated with 5μg/ml lipopolysaccharide (LPS), 100μg/ml thymoquinone (TQ), or 100μg/ml TQ and 1mg/ml Tamiflu, 500nM Galardin or 100ng/ml Pertussis toxin (PTX) for 5 minutes. Cells were fixed with 4% paraformaldehyde for 30 minutes and permeabilized with 0.2% Triton-X in TBS for 5 minutes. Cells were treated with 1:200 rabbit anti-mouse anti-MyD88 and 1:200 rat anti-mouse anti-TLR4 for 60 minutes at 37°C. Cells were washed and treated with goat anti-rabbit AlexaFluor 594 for 60 minutes at 37°C then with rabbit anti-rat AlexaFluor 488 for 60 minutes at 37°C. Fluorescent images were taken using epi-fluorescent microscopy (40X objective) and confocal microscopy. These data are representative of two individual experiments.
<table>
<thead>
<tr>
<th></th>
<th>TLR4</th>
<th>MyD88</th>
<th>Overlay</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td><img src="image1" alt="Control TLR4" /></td>
<td><img src="image2" alt="Control MyD88" /></td>
<td><img src="image3" alt="Control Overlay" /></td>
</tr>
<tr>
<td>LPS</td>
<td><img src="image4" alt="LPS TLR4" /></td>
<td><img src="image5" alt="LPS MyD88" /></td>
<td><img src="image6" alt="LPS Overlay" /></td>
</tr>
<tr>
<td>TQ</td>
<td><img src="image7" alt="TQ TLR4" /></td>
<td><img src="image8" alt="TQ MyD88" /></td>
<td><img src="image9" alt="TQ Overlay" /></td>
</tr>
<tr>
<td>TQ + Tamiflu</td>
<td><img src="image10" alt="TQ + Tamiflu TLR4" /></td>
<td><img src="image11" alt="TQ + Tamiflu MyD88" /></td>
<td><img src="image12" alt="TQ + Tamiflu Overlay" /></td>
</tr>
<tr>
<td>TQ + Galardin</td>
<td><img src="image13" alt="TQ + Galardin TLR4" /></td>
<td><img src="image14" alt="TQ + Galardin MyD88" /></td>
<td><img src="image15" alt="TQ + Galardin Overlay" /></td>
</tr>
<tr>
<td>TQ + PTX</td>
<td><img src="image16" alt="TQ + PTX TLR4" /></td>
<td><img src="image17" alt="TQ + PTX MyD88" /></td>
<td><img src="image18" alt="TQ + PTX Overlay" /></td>
</tr>
</tbody>
</table>
4.6 Cell morphology following treatment with TQ

4.6.1 TQ treatment does not affect cell viability

Propidium iodide and Acridine orange were used to stain HEK-TLR4 cells to test for cell viability after TQ treatment. Cells appearing green under the epi-fluorescent microscope were considered viable whereas those appearing orange or red were considered non-viable. As controls, HEK-TLR4 cells were left untreated or treated with hydrogen peroxide to induce cell death. Untreated cells had minimal death whereas cells treated with Hydrogen peroxide sustained substantial cell death. Cells treated with 200μg/ml or higher concentrations of TQ sustained approximately less than 25% cell death. Cells treated with concentrations of TQ less than 200μg/ml had minimal to no cell death. Cells treated with 500μg/ml of TQ and Tamiflu sustained maximal cell death with very few viable cells viewed via epi-fluorescence microscopy. Only approximately 5 to 10% of cells died when treated with 100μg/ml or 250μg/ml of TQ plus Tamiflu (Figure 30.). Cell viability was similar in BMC-2 cells treated with TQ (Appendix A Figure 39.)
HEK-TLR4 cells were grown on 12mm circular glass slides in medium containing 5% fetal calf serum and 0.8μg/ml G418 for 24 hours at 37°C in 5% carbon dioxide. The medium was removed and cells treated with 200nM Tamiflu for 1 hour or left alone. After removal of all medium from other cells they were left alone (control), or treated with 10μg/ml to 1mg/ml TQ or 0.5mM to 1mM hydrogen peroxide (H$_2$O$_2$) for 1 hour. After removal of treatments, 4μl of Dako mounting was added with 1μl of prepared stain mix containing 1μl of Acridine orange, 1μl of Propidium iodide and 998μl of sterile 1x TBS was added to cells. Fluorescent images were takes using epifluorescent microscopy (40X objective). These figures represent one of two individual experiments.
4.6.2 Morphology of cells affected by TQ treatment

To address the question of whether TQ could potentially induce changes in cell morphology, we treated macrophage cells with either TQ or Tamiflu followed by TQ. The untreated cells remained fairly consistent in shape throughout the experiment although some seemed to diminish in size slightly (Figure 31.). Cells treated with TQ showed morphology similar to the untreated controls throughout the experiment. The cells began aggregating and clumping after a few minutes of TQ treatment. Cells treated with 1mg/ml of Tamiflu quickly became elongated, skinny and dark with very dark ridges or small, round and light in colour. After TQ was added to these cells they began to almost resemble control cells. Most cells treated with 200nM Tamiflu became small, round and light in colour although a few of these cells were elongated similar to the cells with the 1mg/ml treatment of Tamiflu. TQ caused all cells treated with 200nM Tamiflu to enlarge to control size but blebbing started to occur rapidly. All cells remained adherent throughout this study.
BMC-2 cells were grown on a 24 well plate in DMEM medium containing 5% fetal calf serum for 6 hours at 37°C in 5% carbon dioxide. After removal of medium from cells to be treated, 100μg/ml or 1mg/ml or 100μg/ml of Tamiflu was added to the cells. Pictures were taken immediately and every 10 minutes on Fisher Scientific inverted microscope under phase contrast using Micron photo software. After 30 minutes, Tamiflu was removed and replaced with 100μg/ml TQ. This experiment was done in duplicate.
<table>
<thead>
<tr>
<th>Time in min</th>
<th>0</th>
<th>10</th>
<th>20</th>
<th>30</th>
<th>30</th>
<th>40</th>
<th>50</th>
<th>60</th>
</tr>
</thead>
<tbody>
<tr>
<td>Thymoquinone</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(after 30 min)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tamiflu 1mg/ml</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(first 30 min)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tamiflu 200nM</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(first 30 min)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Chapter 5

Discussion

While the therapeutic, anti-inflammatory effects of thymoquinone are numerous and well-documented, evidence presented in this study actually shows a novel pro-inflammatory effect of TQ. This finding is made more interesting by the fact that we and others have shown that black seed oil (BSO) is an inhibitor of LPS-induced sialidase activity suggesting that the crude BSO may be an anti-inflammatory agent (Appendix A, Figure 32.). This anti-inflammatory activity was also apparent with the BSO volatile oil constituent, para-Cymene. TQ is another BSO volatile oil component that was also expected to inhibit LPS-induced sialidase activity. Surprisingly, TQ induced sialidase activity similar to that induced by LPS. This seeming dichotomy may be explained by the small quantity of TQ present in the crude oil, 0.2% w/v, where the overall inhibitory effects of the constituents of BSO are able to mask the pro-inflammatory effects of TQ [71]. Also of interest was that both BSO and P-Cy were unable to noticeably inhibit the sialidase activity induced by TQ treatment (Figure 19 and Appendix A Figure 34.). P-Cy was most likely unable to inhibit TQ even at large ratios due to the fact that it is not strong enough without the other BSO components.

Upon discovery of TQ’s ability to induce sialidase activity in macrophage cells, our research focused on deducing what sialidase enzyme was activated with TQ treatment, what the results of this activation were in vitro and in vivo, and what other enzymes were involved in the activation.

The experimental evidence indicated that TQ was activating Neu4 sialidase. This discovery was supported by the lack of sialidase activation in macrophage cells treated with Neu4
neutralizing antibody and in macrophage cells isolated from Neu4 knockout mice. The ability of TQ to act as an agonist to Neu4, may help us to investigate the importance of this recently discovered sialidase enzyme.

One of the key roles performed by Neu4 is the maintenance of lysosomal structure [12]. Tay-Sachs, gangliosidosis, sialidosis and galactosialidosis are important lysosomal storage disorders that result from sialidase-related deficiency. Tay-Sachs disorders result from the deficiency of hexosaminidase neuroglia cells causing an accumulation of GM2 ganglioside [12]. Treatment of lysosomal storage disorders, such as Tay-Sachs, with Neu4 has been beneficial in restoring normal lysosomal storage [12]. One of many defects in the GLBI gene (encoding β-galactosidase and elastin binding protein) transpires as a genetic disease known as G_{M1}-gangliosidosis that causes a myriad of neurological symptoms. The symptoms, including visceromegaly, skeletal and central nervous system abnormalities, are a result of the accumulation of GM1-gangliosides in the central nervous system [72]. As a consequence of some of these mutations to the GLBI gene, elastogenesis is often impaired due to the genetic relationship between β-galactosidase (GLB1) and elastin binding protein (EBP) [73]. It has been shown that deficiency of GLB1 also affects the functionality of lysosomal Neu1, decreasing its ability to desialylate substrates [74]. It has been found that the presence or absence of EBP may have the same effect on the function of Neu1 at the cell surface. As a result of the EBP deficiency, the function of Neu1 is affected and a build-up of glycoconjugates can be found in the visceral organs of patients with some types of G_{M1}-gangliosidosis [75]. Neu4 has been found to be an important part of GM1 ganglioside metabolism [12]. As Neu4 is not associated with EBP or GBL1, its function may remain stable in this disease. Therefore, treatments that activate Neu4, such as TQ, may be able to overcome such disorders. Other lysosomal storage disorders may also be treatable.
with TQ due to the wide substrate specificity of Neu4. Neu1 deficiency is implicated in a human autosomal recessive inborn error metabolism disease called sialidosis or mucolipidosis I [70, 72]. There are 34 known mutations to the Neu1 gene that result in this disease [44]. Patients with sialidosis have a build up of sialylated glycoconjugates in tissues and urine causing neuropathic or non-neuropathic symptoms depending on the onset of the disease [76]. This disease has also been implicated in deficiency of elastogenesis due to the relationship between Neu1 and EBP [72]. The inherited genetic mutation of the PPCA gene, which codes for a protein that provides stability to the EBP complex, results in a lysosomal storage disease known as galactosialidosis [77]. Due to the lack of PPCA there is also a deficiency in Neu1 related to this disorder [72]. Over-expression of Neu4 has been shown to clear the storage material accumulated in the lysosome in these disorders [13]. Investigations of the ability of TQ to treat Tay-Sachs, gangliosidosis, sialidosis and galactosialidosis should be completed.

Contrary to literature, our research has shown that TQ treatment produces a strong transient pro-inflammatory response in mouse macrophage cell lines, human epithelial cell lines, primary mouse macrophage cells and in vivo in a mouse model. Both cytokine array profiles from in vivo experiments and cytokine mRNA levels showed increase in pro-inflammatory cytokines. TQ was also not able to inhibit the sialidase activity induced by LPS. According to literature, TQ is able to inhibit LPS-induced TNF-α production after treatment for 12 hours [42]. Studies did not show significant inhibition of LPS-induced TNF-α production after 6 hours of treatment with TQ [42]. Our data showed that the TQ-induced increase TNF-α mRNA expression peaks around 4 hours and fades quickly. The production of TNF-α may be exhausted by the 12 hour TQ treatments that were tested in previous studies [42]. Treatment with TQ is also able to induce the production of the mRNA of other pro-inflammatory molecules after 4 hour stimulation.
such as MIP-1β and IL-6. Cytokine production in vivo increased 5 hours after wild type and Neu1 deficient (NeuIn) mice were treated with TQ. This was contrary to LPS treatment in which the cytokine production was reduced in NeuIn mice (data not shown). Mice lacking the Neu4 gene were not subject to the large increase in cytokine production following treatment with TQ. Unfortunately, time course evaluation of in vivo cytokine production with TQ treatment was not an option in this project due to restricted use of animals and cost of each array membrane. Time course determination may be critical in future studies.

NFκB is a transcription regulator which is associated with cytokine production and inflammation [78]. NFκB has multiple subunits including p50 and p65, which form homodimers that respond to pro-inflammatory signals to affect the inflammatory response. The two NFκB homodimers affect the transcription of pro-inflammatory genes in an opposing manner, p50 blocks and p65 stimulates DNA promoters for cytokines [42]. Using immunocytochemistry techniques, the activation and nuclear translocalization on NFκB p65 was seen after 5-10 minute treatments with TQ in HEK TLR4 cells and 10-15 minute treatments in BMC-2 cells. In LPS treated cells, the activation and nuclear localization of NFκB p65 occurred after 45-60 minute treatments. The NFκB p65 activation in the TQ-treated cells occurs much more rapidly than the LPS treated cells and expires quickly as well. This transient activation of NFκB p65 may explain why the cytokine response to TQ treatment occurs quicker than LPS and fades rapidly. Tamiflu, a broad range sialidase inhibitor reduces the TQ-induced NFκB p65 activity. This suggests that TQ-induced sialidase activity is required for subsequent NFκB p65 activation. The time required for NFκB p65 activation and cytokine production by TQ versus LPS treatment may also be due to the difference in sialidase activation between Neu4 and Neu1.
This research gives clues to the mechanism of action and binding site of TQ, which have not been elucidated. Both the Gα subunit of the GPCR and MMP9 have been linked to the TQ-induced sialidase activity (Figures 19 to 22.). Inhibitors of MMP9 including galardin, piperazine and neutralizing anti-MMP9 antibody have been shown to inhibit TQ-induced sialidase and NFκB activation. Human and mouse macrophage cells are known to have elevated levels of Pertussis toxin (PTX) sensitive Gαi2 and Gαi3 G proteins [57]. PTX was able to inhibit TQ-induced activation of human and mouse cells. It is reasonable to speculate that the G protein involved in TQ-induced sialidase activation and subsequent pro-inflammatory response may be Gαi2 and Gαi3. TQ may activate Neu4 through Gαi activation of MMP resulting in sialidase activity (Figure 32.). GPCR activation through MMP has been previously demonstrated in the trans-activation of EGFR [52, 53]. G proteins reside at the cell surface with their associated GPCR and some may be activated by means other than a GPCR ligand [79]. The lack of inhibition of TQ-induced cellular activation by Suramin, a broad range GPCR inhibitor that acts only to uncouple the G proteins from the GPCR complex, suggests that TQ may specifically target Gα activation rather than activating GPCR. We believe that TQ may bind or activate the Gαi subunit of the GPCR which subsequently activates MMP9. The elastase activity of MMP9 is proposed to be important in Neu1 sialidase activation due to the association of Neu1 with elastin binding protein (EBP) [16]. The elastase activity of MMP9 may also be important in Neu4 activation although it is not associated with EBP. MMP9 possesses gelatinase and collagenase enzymatic activities that may be involved in the activation of Neu4. There has also been previous evidence of MMPs activating GPCRs such as the thrombin receptor, PAR-1 [54]. Therefore, we also speculate that TQ may activate MMP9 first resulting in the activation of GPCR Gα subunit and subsequent activation of Neu4 (Figure 33.).
Figure 32. Experimental Model 1: TQ-induced sialidase activity through GPCR G\(\alpha_i\)-MMP9-Neu4

This figure illustrates a possible mechanism of action of the pro-inflammatory effects of TQ. In this model TQ binds or activates GPCR G\(\alpha_i\) subunit which activates MMP9 with subsequent activation of Neu4 sialidase enzyme. The activation of Neu4 results in NF\(\kappa\)B activation and RNA transcription.
Figure 33. Experimental Model 2: TQ induces sialidase activity through MMP9-GPCR Gαi-Neu4

This figure depicts a possible mechanism of action of the pro-inflammatory effects of TQ. In this model TQ activates MMP9 which activates GPCR Gαi subunit with subsequent activation of Neu4 sialidase enzyme. The activation of Neu4 results in NFκB activation and RNA transcription.
Once ligand is bound to its receptor, the activation of the respective receptor often involves a myriad of intermediate activation steps. For example, TrkA and Toll-like receptors require sialidase enzyme activity for activation [9, 16]. Some receptors are even able to be activated in the absence of their natural ligand if the activation steps are triggered. EGFR is a prime example of subsequent receptor activation following GPCR activation [52]. There is no published confirmation of the ability of TLR to be activated in the absence of their natural ligands. There are preliminary data obtained in our lab that TLR4 can be activated with α2,3-specific neuraminidases such as T. cruzi trans-sialidase and α2,3 neuraminidase derived from Streptococcus pneumoniae. We believe that the desialylation of glycosylated residues on the receptor allows for the activation, dimerization, and subsequent internalization of TLR4 [10]. Therefore, it may be extrapolated that TLR4 can be activated in the absence of ligand if sialidase enzymes are activated. Considering TQ is able to induce sialidase activity in the absence of TLR, in the HEK 293 cell line, we do not believe that TQ is a TLR ligand (Appendix A Figure 38.). Any TLR activation would therefore be due to inducing the receptor activation steps, specifically sialidase enzyme activity. TQ is able to activate TLR4 as seen in the co-localization of MyD88 and TLR4 (Figure 27A and B.) As we have previously discussed, the sialidase activity induced by TQ is linked to GPCR Gα subunits and MMP9. TQ may be binding or activating one of these intermediates to induce Neu4 sialidase activity and subsequent TLR4 activation in TLR-expressing cells.

To further confirm the trans-activation of TLR, it has been determined that TQ treatment is unable to induce TLR4 activation in the absence of Neu4 (Figure 28.). It has been previously determined in our lab that Neu1 is the sialidase enzyme linked to TLR4 activation [16]. With TQ treatment the activation of Neu4 seems to be overriding or acting in place of Neu1. This proposed
model could be a novel role for Neu4 (Figure 33.). This is promising evidence that activation of Neu4 may help to treat previously discussed lysosomal storage disorders, sialidosis and galactosialidosis that result from Neu1 deficiencies.
Figure 34. Experimental Model 3: TQ-induced activation of Neu4 leads to subsequent activation of TLR4

This figure represents the possible mechanism of TLR4 activation by TQ. Treatment with TQ results in the activation of Neu4 sialidase enzyme that then cleaves the α2,3 sialic acid from TLR4 glycosylated residues. The de-glycosylation of TLR4 results in dimerization and activation of the receptor. The co-factors including MyD88 are required for TLR4 signal transduction and subsequent activation of NFκB. Typical TLR4 activation by natural ligand, LPS, is a result of receptor desialylation by Neu1, the sialidase bound to TLR4.
LPS-induced inflammatory response results in the up regulation of many cytokines and other pro-inflammatory molecules. One well established marker of inflammation is the increased production of intercellular adhesion molecule (ICAM-1). During the morphology study of macrophage cells treated with TQ, the cells were observed moving and clumping together. This clumping is similar to the LPS-induced ICAM-1 response noted in literature [80]. LPS-induced ICAM-1 is responsible for the adhesion and aggregation of macrophage and neutrophil cells [80]. The presence of ICAM-1 in LPS-induced inflammation is responsible for the infiltration of neutrophil cells and migration of leukocytes to inflammatory sites [81, 82]. The TQ-induced up regulation of adhesion molecules was seen in vivo in the cytokine array study (Figure 16 and 17.). The ICAM may be responsible for the macrophage cell migration seen with TQ treatment. The presence of this LPS-like adhesion molecule response to TQ is evidence of the pro-inflammatory effect of TQ and Neu4 activation of TLR4.

Treatment of cells with TQ results in good cell viability. In agreement with the literature, non-cancerous cells do not experience vast apoptosis when treated TQ. When cells are treated with Tamiflu and TQ, cell viability is affected in a dose dependent manner. Although not tested in this study, cancerous cells appear to have low cell viability after treatment with TQ [22].

TQ has been shown to have anti-cancer properties in a myriad of cancerous cells and models. Treatment with TQ is able to induce apoptosis, reduces cellular growth and mitosis, protein and DNA degradation, or interrupts the mitochondrial membrane in carcinoma, osteosarcoma, fibrosarcoma, leukemia, colon cancer and papilloma infected cells [22]. The mechanism by which TQ is able to produce such extreme anti-cancer reactions has yet to be elucidated. Many pathways have been linked to the anti-cancer properties of TQ including activation of p53, caspases 3, 8 and 9 and the reduction of p16.
Neu3 is an inhibitor of apoptosis that has been found up regulated in human colon cancer [47]. Neu4 has shown an opposing role to Neu3 in this cancer model. The presence of colon cancer is linked to a decrease in Neu4 production. The knock out of the NEU4 gene by siRNA has been linked to decreased apoptosis, and increased invasion and motility of cancerous cells. Colon cancer cells transfected with Neu4 showed increased apoptosis and decreased invasion and motility [47]. TQ is able to induce sialidase activation in cancer cell lines. The sialidase activity in mouse mammary adenocarcinoma cell line, SP1, is highly up regulated with TQ treatment (Appendix A Figure 40.). These results suggest that the anti-cancer properties of TQ may be in part due to its ability to activate Neu4.

The activation of Neu4 may also be linked to the ability of TQ to induce mitochondrial membrane degradation [28]. The long form of Neu4 has been localized to the mitochondria and plays a role in regulating the accumulation of G_{D3} gangliosides within the mitochondria. The accumulation of G_{D3} gangliosides occurs during apoptosis; therefore Neu4 may play a role in apoptosis through the ability to control the accumulation of these gangliosides in the mitochondria. As TQ has an effect on Neu4 activation this may be one way TQ is able to influence apoptosis in cancerous cells.

TQ induces the production of various cytokines. Often the increased production of cytokines is associated with pro-cancerous properties, such as motility and invasion, although there are some examples of amplified cytokine production resulting in cell cycle arrest and apoptosis in cancerous cells. Tomihara et al. found that the increased production of IFN-γ was responsible for the anti-cancer effects seen with CD40 ligand treatment [83]. Other groups have found benefits of IFN-γ in cancer treatment [84]. The increased production of INF-γ induced by TQ may also have anti-cancer effects.
TQ-induced activation of MMP9 may also play a role in its ability to act as an anti-cancer agent. A novel role has been revealed for MMP9 in the induction of apoptosis. It has been found that the inhibition of MMP9 results in decreased apoptosis in spinal cord injuries [85]. The role of MMP9 in apoptosis may elucidate one mechanism in which TQ is able induce apoptosis in cancerous cells.

Treatment with TQ may affect many mechanisms and pathways that induce anti-cancer effects. Our research has suggested a role for cytokines, MMP9 and sialidase activity in apoptosis and cell cycle arrest caused by TQ treatment in cancer cells. TQ has been shown to be effective at treating many other diseases as well.

TQ has been studied for treatment response in chemically-induced diabetic hamster and rat models. TQ abolishes high blood glucose levels, hyperglycemia and hypoinsulemia that is present in these animals after the induction of a diabetic state [38, 39]. It is believed that the benefits of TQ come from the ability to decrease gluconeogenesis. Preliminary research conducted in our lab places the insulin receptor under the influence of sialidase enzyme activity similar to TLR and TrkA. This was revealed by the significant sialidase activity upon treatment of cells with insulin (data not shown). These data suggest that TQ-induced Neu4 sialidase activation could have a role in the anti-diabetic properties associated with TQ treatment.

Overall, TQ had many unexpected properties including the increase of cytokine production and activation of TLR4 upon treatment. This study gives us a novel pathway in which TQ may be acting and a hint at where the primary target of TQ may be. TQ treatment may have future biological roles as a therapeutic agent for various types of cancer and diabetes.
References


128


Appendix A
Supplemental Data

Figure 35. Ligand-induced sialidase activity inhibited by black seed oil

BMC-2 cells were incubated on 12 mm circular glass slides in medium containing 5% fetal calf serum for 24 hours at 37°C. After removal of medium, 0.2mM 4-MUNANA (4-MU) substrate in Tris buffered saline pH 7.4 was added with mounting medium (Dako) to cells alone (Control), with 333μg/ml LPS, or 250μg/ml LPS and various doses of black seed oil (BSO). The substrate was hydrolyzed by activated sialidase to give free 4-methylumbelliferone which has a fluorescence emission at 450 nm (blue color) following excitation at 365 nm. Fluorescent images were taken at one minute time intervals using epi-fluorescent microscopy (40X objective). These data represent one of five individual experiments.
Figure 36. Ligand-induced sialidase activity inhibited by BSO component para-Cymene

BMC-2 cells were incubated on 12 mm circular glass slides in medium containing 3% fetal calf serum and 5% horse serum for 24 hours at 37°C. After removal of medium, 0.2mM 4-MUNANA (4-MU) substrate in Tris buffered saline pH 7.4 was added with mounting medium (Dako) to cells alone (Control), with 167μg/ml Bombesin, or 125μg/ml Bombesin and various doses of para-Cymene (p-Cy). The substrate was hydrolyzed by activated sialidase to give free 4-methylumbelliferone which has a fluorescence emission at 450 nm (blue color) following excitation at 365 nm. Fluorescent images were taken at one minute time intervals using epi-fluorescent microscopy (40X objective). These figures represent one of six individual experiments.
Figure 37. BSO and p-Cy do not inhibit TQ-induced sialidase activity

BMC-2 cells were incubated on 12 mm circular glass slides in medium containing 3% fetal calf serum and 5% horse serum for 24 hours at 37°C. After removal of medium, 0.2mM 4-MUNANA (4-MU) substrate in Tris buffered saline pH 7.4 was added with mounting medium (Dako) to cells alone (Control), with 250μg/ml TQ, p-Cy, BSO, LPS, LPS and p-Cy, BSO or TQ, or TQ and p-Cy. The substrate was hydrolyzed by activated sialidase to give free 4-methylumbelliferone which has a fluorescence emission at 450 nm (blue color) following excitation at 365 nm. Fluorescent images were taken at one minute time intervals using epi-fluorescent microscopy (40X objective). These data represent one of three individual experiments.
Figure 38. TQ-induced sialidase activity in the absence of TLR expression in HEK 293 cells

HEK293 cells were incubated on 12mm circular glass slides in medium containing 3% fetal calf serum and 5% horse serum for 24 hours. After removal of medium, 0.2mM 4-MU sialidase specific substrate in Tris Buffered saline pH 7.4 was added with Dako mounting medium to cells alone, with 167μg/ml Bombesin, or 333μg/ml TQ. The substrate was hydrolyzed by activated sialidase enzymes to give free 4-methylumbelliferone which has a fluorescent emission at 450nm (blue) following excitation at 365nm. Fluorescent images were acquired at one minute intervals using epi-fluorescent microscopy (40X objective). These figures are representative of one of five experiments.
Figure 39. Cell viability of TQ treated BMC-2 macrophage cells

BMC-2 cells were grown on 12mm circular glass slides in medium containing 3% fetal calf serum and 5% horse serum for 24 hours at 37°C in 5% carbon dioxide. The medium was removed and cells were treated with 5% BSO or p-Cy for 30 minutes or left alone. After removal of medium from other cells they were left alone (control), or treated with 1mg/ml TQ or serum free medium for 30 minutes. After removal of treatments, 4μl of Dako mounting was added with 1μl of prepared stain mix containing 1μl of Acridine orange, 1μl of Propridium iodide and 998μl of sterile 1x TBS was added to cells. Fluorescent images were taken using epi-fluorescent microscopy (40X objective). These data represent one of four individual experiments.
Figure 40. TQ-induced sialidase activity in non-metastatic infiltrating mouse mammary adenocarcinoma (Sp1) cells

Non-metastatic infiltrating mouse mammary adenocarcinoma (Sp1) cells were incubated on 12mm circular glass slides in medium containing 10% fetal calf serum for 24 hours. After removal of medium, 0.2mM 4-MU sialidase specific substrate in Tris Buffered saline pH 7.4 was added with Dako mounting medium to cells alone, with 3.8mg/ml Mycobacterium (MYCO), 333μg/ml TQ, or 250μg/ml TQ and 250mg/ml Tamiflu, 250μg/ml Galardin, or 25ng/ml PTX. The substrate was hydrolyzed by activated sialidase enzymes to give free 4-methylumbelliferone which has a fluorescent emission at 450nm (blue) following excitation at 365nm. Fluorescent images were acquired at one minute intervals using epi-fluorescent microscopy (40X objective). Fluorescence in pictures was analyzed by averaging the red, green and blue channels of 50 points proximal to cells using ImageJ software. Mean fluorescence was graphed using GraphPad Prism software. These data represent one of three individual experiments.