THESIS

METRONIDAZOLE NEUROTOXICITY

Submitted by

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In partial fulfillment of the requirements

For the Degree of Master of Science

Colorado State University

Fort Collins, Colorado

Fall 2021

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ABSTRACT

METRONIDAZOLE NEUROTOXICITY

Metronidazole is a broad-spectrum antibiotic approved for clinical therapeutic use in veterinary and human medicine. Although the literature has reported neurotoxic unintended side effects with the use of this drug, these incidences occur in less than 1% of human cases making this instance rare. The mechanism of this neurotoxicity has not been fully elucidated, nor the susceptible population identified. We explore in this work that these susceptible populations are humans and animals with concurrent localized and/or systemic inflammation. Some proposed mechanisms are axonal swelling with increased water content due to toxic injury, vascular spasm with mild reversible localized ischemia, modulation of the gamma-aminobutyric acid (GABA) receptors within cerebellar and vestibular systems, RNA binding with inhibition of protein synthesis, and axonal degeneration. While these mechanisms offer some insights into the neurotoxicity, we propose a novel connection between cholesterol inhibition and the reductive activation of metronidazole resulting in poor glial myelination that explicates low dose neurotoxic clinical outcomes in vulnerable humans and animals with the use of this drug. In order to investigate this, we have implemented physiologically based pharmacokinetic computational models of a human, equine, murine, and rabbit with metronidazole exposure. Furthermore, in combination with computational techniques, we assess cellular and molecular analyses to address this neurotoxicity in a primary murine glial cell model. Additionally, we use liquid chromatography and mass spectrometry work in order to

address the reductive activation of metronidazole. We then ask if inflamed glia are more susceptible to metronidazole-dependent neurotoxic outcomes. With these data, we offer insight into this elusive mechanism and will aid human and veterinary literature in a way that improves the quality of life of affected patients and better predicts populations vulnerable to this neurotoxicity.

ACKNOWLEDGEMENTS

I would first like to thank my mother and father for believing in me and constantly pouring all of their love and support into me all of these years. To my mother, thank you for all of your love, your support, your long talks, and the way you never hesitate to come to visit me no matter how old I get or what state I live in. I cherish every single moment that we speak even when we just sit on the phone in silence because there's nothing to say (i.e., I keep bothering you at work- hehe). You always make time and space for me to say the things I need to say, and I can't begin to tell you how much it means to me. My heart is full just thinking of your love. Your passion for what you love and what you do never goes unnoticed and I'm grateful to have the extraordinary privilege to experience a motherly love from someone as remarkable, unselfish, kind, and caring as you. I can only dream of attaining a heart and mind like yours. I love you with *all* my heart. To my father, thank you for supporting me, loving me, all of your (not-so-funny) jokes, and lunch dates at Popeye's. I am wildly appreciative of just how incredibly deeply you care for me and how it's consistently demonstrated in your actions, thoughts, and words. Your thoughtfulness goes too deep for me to describe here with mere words. I wholly admire you as a person and, most importantly, as my (*#1*) father. I cherish having someone as phenomenal as you in my life and I love you with *all* my heart. Even though nothing else ever goes right in my life, I seem to have hit the jackpot for parents. And I can definitely live with that. I've truly enjoyed how my relationship has grown with each of you over the years and deeply appreciate the way you've both allowed me to unapologetically be myself and unflinchingly support that in any which way you each individually know how. I

am endlessly grateful. To my sister, thank you for all your love, unwavering support over the years, and always picking up the phone when I call. And *especially* so for always being there to do a virtual raid battle with me in Pokémon Go. You are my kind, caring big sister and I am beyond lucky to feel so constantly cherished by you (and I'm also lucky to have someone who shares my deep passion of becoming a true Pokémon Master- *another jackpot for me*). I cherish you and love you so much. To my Nana, thank you for all of your endless love, prayers, and support of me. I will forever and always treasure our time together when you visit me (no matter what state I'm in) and I am grateful to get the chance to experience a love as rich and deep as yours. I love you so much. To my Grandma and Grandpa, I equally thank you both for your great love and support of me. And I can't wait to visit you again in New York. I love you both very much. All, I couldn't have completed this degree without each of you. Thank you.

I would also like to especially thank my advisors and my committee members for their *enormous* support of me in my learning of what it means to do research. Dr. Moreno, thank you for your *endless* support, not hesitating to take me on as a student, and doing anything and everything you could to allow me to pursue my own project and passions. You've been a great mentor and guide and I'm deeply appreciative I've had the chance to work so closely with such an astounding person. Dr. Legare, thank you for absolutely everything you've done for me and all of your support over the years. You have also been such a strong and compassionate advisor and mentor in my life, and for that, I'm grateful. (I can't wait for the next Knit n' Sip). Drs. Bouma and Tjalkens, it has been a true pleasure working with you both over the past few years and I'm deeply grateful for all of your genuine support of me. I also would not be here without you all. Thank you.

Finally, an honorable mention goes out to my dear friends, Mandy and Elisa, and mentors, Tim, Kas, and Vinny, for all of their support as well as Zwei for single-handedly supporting all of us in the Tox program over the years.

DEDICATION

This work is dedicated to the late William H. Hanneman. Words cannot express my gratitude to you for all of the support and unwavering belief you had in me during our short time together.

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Introduction

1.1 Brain anatomy and function

The cornerstones of numerous bodily functions ranging from processing thoughts to complex physical movements can be attributed to two major systems in the body known as the central and peripheral nervous systems. Within the central nervous system (CNS) lay two major organs, the brain, and spinal cord. The brain is made up of individual constituents known as the cerebrum, cerebellum, and brain stem. The cerebrum is the largest part of the brain and is composed of left and right hemispheres. Most notably, the cerebrum functions to decipher things such as touch, speech, reasoning, emotions, and fine control of movement (Britannica 2020). The cerebellum is located at the base of the brain and is in control of coordinating muscle movements and balance. Finally, the brainstem functions as a critical part of the CNS by transmitting messages between the cerebrum, cerebellum, and spinal cord. It is also responsible for automated functions such as breathing, heart rate, digestion, and more. Furthermore, within the brain, there are deeper structures such as the hypothalamus, pituitary gland, pineal gland, thalamus, basal ganglia, and limbic system. Briefly, the hypothalamus is largely known for secreting hormones and these hormones play large roles in thirst, hunger, sleep, regulation of body temperature, blood pressure, and emotions. Next, the pituitary gland controls the endocrine glands. It also secretes hormones, and these hormones play major roles in bone development, muscle growth, and responses to stress. The pineal gland is notable

for its role in regulating sleep cycles and the body's internal clock through the secretion of a hormone called melatonin. The thalamus functions to alert the body to pain sensations and even plays a role in memory. The basal ganglia are a group of subcortical nuclei that is responsible for fine motor movements and does this through working with the cerebellum. Finally, the limbic system is known as the center of emotions, learning, and memory. Within the brain exists hollow cavities filled with fluid known as ventricles. Within ventricles exists a structure called the choroid plexus that produces a clear fluid known as the cerebral spinal fluid (CSF). CSF acts as a cushion for the brain and helps protect it from any injuries (Britannica 2020). The other major system, the peripheral nervous system (PNS), is most notably what connects the brain and spinal cord to all the various tissues and organs within the body. The PNS can be further divided into two components, the sensory and motor systems. The sensory system, also known as the afferent component, relays information to the central nervous system from the body and environment. Conversely, the motor component, also known as the efferent component, carries information from the CNS to tissues such as the muscles (Krassioukov 2002). Within the PNS exists receptors that function as transducers. Essentially, transducers work by converting various stimuli, such as mechanical stimuli, into electrical impulses. Two transducers provide signals related to the mechanical state of the muscles. One is the neuromuscular spindles that contain a fibrous capsulated striated fiber which is coiled around neural endings that transfer signals to the alpha motor neurons of the anterior horn of the spinal cord through a fast-monosynaptic junction. The second transducer is the Golgi organ, a force-related transducer responsible for the inverse myotatic reflex which is a protective reflex against muscle overexertion. These two complex mechanisms

work in unison to allow mammals to have movement. Additionally, the body employs two levels of motor control, conscious versus unconscious control of movement. One level involves movement without the use of muscles thus allowing the body to move around a point in space consciously chosen by the subject. The other level involves working unconsciously and this motor control accounts for a majority of the motor circuits. Once a decision or action is made a circuit goes from the premotor area to the motor thalamus then to the lateral cerebellum where the motor program is organized and back to the motor cortex to a group of required muscles reaching through the corticospinal tract to activate motor movement. A similar circuit connects the cortical area to the basal ganglia where all the storage for motor programs lies (Scarbino et al. 2006).

Communication throughout the body consists of largely sensory inputs. Sensory inputs are largely from sensory organs that connect the brain to specific information such as vision, hearing, touch, and smell. However, communication can also be maintained throughout the body through the endocrine, or hormone, system. Both of these systems work closely together to regulate the body. Within the endocrine system, endocrine glands produce chemicals known as hormones into the blood to be circulated throughout the body that function as chemical messengers. This can all be accomplished in numerous ways. Generally, organs within the central nervous system produce hormones directly from nerve cells and are considered prominent connectors between the endocrine and nervous systems. For example, the hypothalamus which plays a role in hunger receives signals from outside stimuli such as food to release hormones such as corticotropin-releasing hormone (CRH) which regulates carbohydrate, protein, and fat metabolism (Hiller-Sturmhöfel et al. 1998 and Scarbino et al. 2006).

Overall, numerous systems within the body work together to accomplish everything from basic biological maintenance to much more complicated functions.

1.2 The role of glia

Neurons and glia are the driving force of brain function and subsequent body functionality. Neurons and glia work together in a close molecular relationship to achieve various functions of the mammalian body system. The main duties of neurons are to generate and propagate electrical and chemical signals and are therefore integral to communication within the body. Glia exist largely to support neurons and mainly do this by modulating neuronal function and signaling. Multiple types of glia exist and localize to either the CNS or PNS. Within the CNS, the distinctive sorts of glia include astrocytes, oligodendrocytes, ependymal cells, radial glia, and microglia. Meanwhile, in the PNS, Schwann cells, satellite cells, and enteric glia are the most prevalent types (Brophy et al. 2009).

Astrocytes are the most predominant glial cell type in the CNS. Fine processes of astrocytes form connections with neurons by surrounding them in subcellular specializations especially at synapses, or end feet that contact vasculature. Through this, astrocytes play a role in regulating local blood flow through the release of neurotransmitters from neurons that activate receptors on adjacent astrocytes triggering a calcium response that promotes the release of vasoactive substances in the astrocytic feet in contact with blood vessels. To provide energy to neurons, astrocytes additionally take up glucose from the blood and convert it to lactate. Furthermore, astrocytes buffer extracellular potassium concentrations with increased levels of activity that depolarize neuronal membrane potentials to pathological states. They also play a role in local

oxygenation and water homeostasis. Moreover, astrocytes also help control neuronal excitability, regulate synapse formation, function, and plasticity, and overall brain homeostasis by regulating brain volume in potassium concentrations. In response to various disease pathologies and injuries of the brain, astrocytes go through a defensive process called astrogliosis. During astrogliosis many changes occur in astrocytes such as cytoskeletal hypertrophy and in addition to this, many will express extracellular matrix molecules. These changes can ultimately cause a dense glial scar that can promote healing by forming a protective barrier for damage. Contrary, this scar can be full of extracellular matrix molecules that inhibit axon regeneration (Brophy et al. 2009).

Another important component of neuronal function is myelin. Myelin, which is made up of proteins and phospholipids, surrounds the axons of neurons creating a sheath that helps increase the speed of nerve impulses. This is largely produced by oligodendrocytes in the CNS and Schwann cells in the PNS. Typically, within the CNS, one oligodendrocyte will contribute to making myelin on multiple axons whereas Schwann cells in the PNS will only contribute to one axon at a time. This means multiple Schwann cells will line a single axon in a long line. Furthermore, along the myelin sheath, there exist intermittent breaks that are called nodes of Ranvier. The four major domains of the nodes of Ranvier are paranodes, juxtaparanodes, and internodes. These make up different types of axons in both the peripheral and central nervous systems. Nodes are defined by high-density clustering voltage-gated sodium channels which regenerate and propagate action potentials. Myelinated axons are completely isolated from the extracellular space except at the nodes of Ranvier therefore myelinating glia must provide metabolic support to axons (Rasband 2016).

Finally, microglia serve largely in a role as the resident immune cell. After an injury microglia will migrate to the sites of injury and undergo dramatic changes in shape and protein expression, release cytokines, and phagocytose debris and dead and dying cells. Furthermore, microglia can remove dysfunctional synapses. Additionally, some research has demonstrated that microglia also play a role in normal development and that deficits in impaired learning can occur with their loss or dysfunction (Rasband 2016).

1.3 Neurotoxicity

Neurotoxicity is a broad term that refers to the direct or indirect effect of a drug or molecule that disrupts the nervous system of mammals. When a substance alters the normal functionality of the nervous system in a way that causes permanent or reversible damage, it is considered neurotoxic. Some molecules can have a direct effect on neurons while others can interfere with metabolic processes involved in the nervous system. These injuries can be sudden and short-lived or slow and chronic and likewise can either cause acute and/or permanent deficits (Spencer et al. 2014). Many interactions between neurons in glia can be disrupted by neurotoxins. For example, the food-associated mycotoxin ochratoxin A inhibits glutamate uptake by astrocytes which then increases extracellular glutamate, excitotoxicity, and neuronal loss. Myelin-producing cells are also susceptible to substances that disrupt the synthesis of myelin components. For example, diphtheria toxin inhibits Schwann cell protein synthesis and causes primary demyelination in peripheral nerves and subsequent oligodendrocyte demyelination as well. Generally, the nervous system is protected from direct exposure to chemicals in the bloodstream and the CSF by the blood-brain, nerve, and CSF regulatory interfaces. The blood-brain-barrier is a well-known, highly selective, and semipermeable border made of endothelial

cells that functions to prevent various chemicals from the circulating blood to cross into the extracellular fluid in the CNS (Daneman et al. 2015 and Ballabh et al. 2004). However, neurotoxins can sometimes unintentionally pass this selective border and cause minor adverse clinical effects such as confusion or more serious outcomes such as seizures. Ultimately, the degree of neurotoxicity depends on a multitude of factors this includes dose, duration of exposure, time of exposure, genetic makeup, the chemical structure of the toxin, and more.

As mentioned previously, molecular level neurotoxicity can manifest in DNA damage, reactive oxygen species (ROS), demyelination, inflammation, mitochondrial dysfunction, protein aggregation, and more. In DNA damage-induced toxicity, toxicants can interact with DNA and cause double and single-strand breaks. Ethanol is a well-known neurotoxicant that can contribute to both DNA damage and ROS production (Kruman et al. 2012). Ethanol is oxidized by alcohol dehydrogenase (ADH), cytochrome P450 2E1 (CYP2E1), and catalase enzymes and through this mechanism of biotransformation can cause an increase in reactive oxygen species, and this can, in turn, can cause damage to DNA. One form of DNA damage can manifest as DNA adducts; DNA adducts block translation of DNA synthesis catalyzed by various DNA polymerases and induce mutations (Hetman et al. 2010). While not as common, demyelination can be another side effect of certain drugs. Pentylenetetrazol is an epileptic seizure-inducing drug with a hallmark for demyelination (You et al. 2013). Pentylenetetrazol is a drug that was previously used as a circulatory and respiratory stimulant and also found useful for depression. Its unfortunate side effects of convulsions and seizures rendered it phased out in 1939 and finally recalled by the FDA in 1982. Now it is useful in experimental studies

as a way to invoke seizures for research use. As stated previously, the myelin sheath is important for the installation of axons of neurons. Oligodendrocytes are the main contributors of myelin to the myelin sheath. Pentylenetetrazol (PTZ) demonstrated myelin sheath damage of the hippocampus and cerebral cortex in the early phases of epileptic seizures (You et al. 2011). Clinically prescribed drugs are not the only causes of neurotoxicity, environmental chemicals can also play a role in adverse drug outcomes. For example, organophosphates (OP) are an organic group of synthetic compounds developed in the early 20th century as insecticides. The principle mechanism of this insecticidal activity is inhibition of acetylcholinesterase (AChE), an enzyme that is conserved across species, which catalyzes the hydrolysis of the neurotransmitter acetylcholine to conclude cholinergic neurotransmission. A large hallmark of this neurotoxicity, other than its cholinergic effects, is neuroinflammation. Depending on the level of exposure, type of OP, and duration of exposure, the neuroinflammatory response can be greatly varied. Inflammation is a term used to describe complex cellular processes that involve the upregulation of proinflammatory cytokines, chemokines, macrophage activation, and leukocyte infiltration of injured tissue (Guignet et al. 2019 and Proskocil et al. 2013). Neuroinflammation in particular specifically refers to an immune-mediated glial cell propagated response organized within the nervous system in response to brain injury or systemic inflammation. In response to inflammation in the central nervous system, astrocytes transform into a reversible, reactive form called astrogliosis which entails the increased expression of cytoskeletal proteins, glial fibrillary acidic protein (GFAP), and vimentin. In response to neurotoxin exposure, there is a marked increase of GFAP and amoeboid microglia in the piriform cortex, amygdala, and hippocampus. Another well-

known initiator of neuroinflammation is an endotoxin/exohormone found in the outer membrane of gram-negative bacteria called lipopolysaccharide (LPS; Marshall 2005). The target of LPS is toll-like receptor (TLR) 4 and through this, it activates downstream pathways that ultimately result in the induction of many pro-inflammatory genes (Batista et al. 2019). Mitochondria are the powerhouses of our cells and responsible for generating energy through adenosine triphosphate (ATP) and play a critical role in apoptosis signaling cascades. Mitochondria are the only subcellular structure other than the nucleus to contain DNA, however, this DNA is not protected by histones as in the nucleus. This leaves mitochondrial DNA exposed and without a physical shield to protect it from free radicals. Unsurprisingly, mitochondrial damage is primarily caused by ROS, however, surprisingly, it is mainly through the ones generated by mitochondria themselves. The majority of ROS is generated by complex I and III through the release of electrons by NADH and FADH into the electron transport chain (ETC) (Neustadt et al. 2008). Psychotropic medications can directly inhibit mitochondrial DNA transcription of ETC complexes, cause damage through other ETC components, and/or inhibit enzymes required for any of the steps of glycolysis and beta-oxidation. Indirectly, these drugs may also increase the production of free radicals, decrease endogenous antioxidants such as glutathione, and/or deplete the body of nutrients required for the creation of mitochondrial enzymes. Finally, a well-known process of protein aggregation is the aggregation of α-synuclein. Although the actual function of α-synuclein is not well understood, it is a protein that largely resides in the brain and is found at the tips of neurons in special structures called presynaptic terminals (Jiang et al. 2016). Presynaptic terminals release chemical messengers called neurotransmitters which aid in communication between neurons. As

with everything mentioned previously, protein dysregulation is just one of many mechanistic results of neurotoxicity. One form of this, protein aggregation, can occur alongside any of the other aforementioned neurotoxic outcomes, and alternatively, drugs can induce any one or a cascade of toxicities. A drug that is known to specifically induce this is called staurosporine. Staurosporine is an antibiotic commonly used in research to induce apoptotic cascades in cells. It was originally isolated from Streptomyces staurosporeus and mainly functions through the inhibition of protein kinases and ultimately induces apoptosis in various cells. However, because there is a lack of specificity with this, it is now mainly used for research purposes. This further demonstrates the variety of ways exogenous chemicals can affect the biology of organisms on a molecular level and subsequently contribute to toxicological outcomes.

In a complementary manner, these minute molecular level modulations can ultimately manifest as either minor or major clinical neurotoxic phenotypic outcomes. Examples of this are ataxia, blindness, numbness, loss of memory and intellect, delusions, headaches, behavioral problems, and more. Many medications describe unintended adverse outcomes as side effects with normal therapeutic use. For example, as a central nervous system depressant, barbiturates have been used in a variety of manners as a short-term treatment for anxiety, insomnia, seizures, and acute migraines (Eaddy 2013). However, due to their high toxicity profile, they have largely been replaced with benzodiazepines as a more preferred therapeutic but long-acting barbiturates are still used as anticonvulsants. In humans and animals (Williams et al. 2011), barbiturates can cause ataxia, seizures, and have an increased incidence of addiction (Nobay et al. 2014). In the brain, γ-Aminobutyric acid (GABA) is the primary inhibitory neurotransmitter

and thereby functions to reduce neuronal excitability. Barbiturates elicit their effects by binding to GABA receptors and potentiating this inhibitory effect, hence its action as a CNS depressant. It can also instigate inhibitory effects on glutamate receptors which furthers this depressant effect since glutamate functions as an excitatory neurotransmitter. The main cause of toxicity is due to its influence on chloride ion channels allowing them to open for longer durations at GABA receptors resulting in chloride influx and membrane hyperpolarization which suppresses excitability (Brewer et al. 2009). Drug-induced peripheral neuropathy (DIPN) is a term coined to describe damage to nerves in the peripheral nervous system by a chemical used for treating, prevention, or diagnosis of a disease and includes optic neuropathy (Vilholm et al. 2014). Blindness is a type of optic neuropathy that can also occur with the use of some drugs. For example, the ingestion of methanol can induce severe irreversible bilateral toxic optic neuropathy. In the late stages, this injury of the optic disc can become necrotic and in some cases lead to complete blindness (Peragallo et al. 2013). Other peripheral neuropathies can include numbness and/or paresthesia. While various drugs can cause this, two notable ones are bortezomib and thalidomide which are used in the treatment of myeloma (Ludwig et al. 2018). Bortezomib does this through being mitochondrial calcium release and subsequent activation of the apoptotic cascade and interference with microtubule stabilization (Jones et al. 2020). While thalidomide may have a relation to antiangiogenic activities as a potential explanation for this DIPN (Delforge et al. 2010). In elderly populations, it is more likely that you will find drug-induced cognitive impairment. Older populations of people additionally tend to take multiple medications for extended periods. It is unlikely that we can differentiate the cause of drug-induced cognitive

impairment due to it being multi-factorial. Psychoactive drugs, narcotic agents, anticonvulsants, tricyclic antidepressants, and benzodiazepines can all contribute to causing delirium or exacerbating dementia (Moore et al. 1999). Another phenomenon called medication-overuse headache is something that is a common worldwide public health problem. Ironically, this is largely caused by over-the-counteract medication (Kristoffersen et al. 2014). Finally, behavioral problems can occur with the use of drugs, notably, behavioral disinhibition which explicates loss of restraint over some form of social behavior (Bond 2012). Tricyclic antidepressants also can play a role in this phenomenon as well as serotonin reuptake inhibitors. This has reportedly led to akathisia, suicidal urges, agitation, hyperactivity, and mania (Bond 2012).

1.4 Biotransformation in the brain

All of these molecular and clinical outcomes of neurotoxicity that have been introduced are mediated by the biotransformation of chemicals. Biotransformation is a process by which substances in the body are changed, through chemical reactions, from one chemical to another and, in a simpler definition, describes a subset of metabolic functions of cells, i.e., metabolism. Both endogenous and exogenous chemicals are subject to this biological process and it plays a significant role in the degree to which a toxicant can elicit its effects. More specifically, in the case of exogenous chemicals, i.e., drug metabolism, the body aims to de-toxify by making a more water-soluble product that is capable of being excreted through the urine. Enzymes are chemicals that catalyze these reactions and can regulate the rate at which it occurs. Enzymatic activity, abundance, localization, and functionality can drastically alter intended outcomes and lead to therapeutic and/or adverse effects. Common enzymes that reside in the brain are

monoamine oxidases (MAO), AChE, and various cytochrome P450 enzymes. MAO catalyzes the oxidative deamination of dopamine and serotonin, two prominent neurotransmitters in the brain. Cytochrome P450 enzymes are extremely abundant in the brain and liver. They comprise many different types of enzymes with various functions such as demethylation, mono-oxidations, hydroxylation, and more. They are implicated in more than 70% of all toxicological drug metabolisms. Using positron emission tomography (PET) imaging, Holland et al. were able to measure the abundance of these enzymes in the brain (Holland et al. 2013). Cytochrome P450 2D6 is an enzyme involved in the biosynthesis of brain molecules such as dopamine and 5-hydroxytryptamine and is also a good example of how genetics can influence chemical toxicity profiles. For example, 7% of Caucasians have a mutation in the CYP2D6 gene that results in them being poor metabolizers of drugs or chemicals that biotransform via this enzyme due to reduced levels in the brain and liver (Ferguson et al. 2011). This can result in less active metabolites and/or prolonged side effects of certain drugs.

In some sense, all chemicals have the potential to be toxic. The dose, duration and frequency of exposure, and route of exposure heavily determine toxicological outcomes of chemicals. A notable application of this would be the different mechanistic consequences of poisons and venoms. When injected, some snake venoms contain enzymes that catalyze reactions in response to present proteins in the blood and subsequently form coagulates. However, when ingested, those same enzymes are exposed to dissimilar proteins that reside in the gastrointestinal tract nullifying this reaction and allowing them to be broken down by stomach acid. In contrast, poisons have the opposite effect. This emphasizes the importance of two main toxicological principles,

enzyme availability and exposure routes. Toxicants can be endogenous, originating from inside the body, as well as exogenous. An example of an endogenous toxicant, peroxynitrite (PXN), elicits its effects through an oxidative stress-related mechanism. PXN is a product of nitric oxide and superoxide radicals, both natural occurrences in cellular metabolism (Tjalkens et al. 2017). Superoxide radical anion is the univalent reduction of oxygen and ubiquitously expressed. Nitric oxide is formed by nitric oxide synthases (NOS) and is a radical involved in neurotransmission and cellular signaling. The product of these two, PXN, is known for being a strong oxidizer and nitrating agent. Due to this, cytotoxic events can occur within the cell. For example, PXN-mediated oxidation can oxidize the mitochondrial membrane and facilitate the release of pro-apoptotic factors, and initiate necrosis. Further, PXN can mediate nitrosylation of Hsp90 and induce motor neuron death. It can also contribute to vascular degeneration and aging as well as protein tyrosine nitration resulting in 3-Nitrotyrosine (Radi 2013). Additionally, protein tyrosine nitration has been linked to pathological diseases such as Parkinson's Disease. Numerous reagents can cause neurotoxicity through unintended mechanisms. Some examples of this are antihistamines, anticonvulsants, pain killers, and even antibiotics. Nonetheless, ultimately, biotransformative reactions can play a large determining role in whether or not a chemical will produce off-target effects that result in neurotoxicological outcomes.

1.5 Antibiotics

Antibiotics are stapled drugs in medicine. The first antibiotic was discovered by Alexander Fleming on accident. He created penicillin from a fungus, *Penicillin notatum*, that contaminated an uncovered culture plate of Staphylococcus. Antibiotics can be naturally derived, as Fleming did, or synthetically derived. The primary use of antibiotics

is to destroy bacterial infestations of mammalian systems. They can do this through two general mechanisms, in a bactericidal or bacteriostatic approach. Bactericidal antibiotics work by killing bacteria (Pankey et al. 2004) while bacteriostatic antibiotics primarily inhibit metabolic processes and stop bacterial growth without a decrease in bacteria numbers, unless given in high enough concentrations (Wald-Dickler et al. 2018). There are multiple classes of antibiotics and some can span multiple uses and are generally considered to be broad-spectrum. Broad-spectrum refers to the capability of an antibiotic to act upon both gram-negative and gram-positive bacteria (Ory et al. 1963). These two major classifications refer to the capability of the cell wall of bacteria to take up a particular stain. Bacterial cell walls that are thick and contain peptidoglycan can retain the primary crystal violet stain and are therefore classified as gram-positive. With the addition of ethanol, thinner cell walls, without peptidoglycan, are not able to retain the crystal violet stain and are stained with the pink or red counterstain known as safranin or fuchsine (Beveridge et al. 1983). These bacteria are classified as gram-negative and those that do not fall into either of these categories are sometimes classified as gram-variable or gram-indeterminate. Additional classifications can involve requirements of oxygen, i.e., anaerobic or aerobic. Drugs like metronidazole, nystatin, micafungin, and terbinafine offer additional support by being anti-fungal in addition to their anti-bacterial properties. In general, these drugs are known as anti-microbials.

Adverse outcomes attributed to antibiotics include ataxia, diarrhea, nausea, dermatitis, headache, respiratory failure, and anemia. Azoles like metronidazole work by being non-competitive inhibitors of the fungal enzyme lanosterol 14-α-demethylase which is a rate-limiting enzyme in the fungal biosynthetic pathway of ergosterol, a sterol that

mimics cholesterol in animal cells. Polyene drugs like nystatin bind ergosterol creating a complex that creates pores in the cell membrane leading to cell leakage and death. Echinocandins such as micafungin inhibit the fungal beta-1(1,3)-D-glucan synthase which synthesizes beta-(1,3)-D-glucan, a component of fungal cell walls, leading to cell death. Finally, allylamines like terbinafine inhibit the rate-limiting enzyme squalene epoxidase which synthesizes precursors to ergosterol and results in the loss of cell membrane integrity (McKeny et al. 2020).

Well-known neurotoxic antibiotics are metronidazole, streptomycin, amoxicillin, cefazolin, and meropenem. While some mechanisms of neurotoxicity are largely unclear, drugs such as streptomycin act by inhibiting the presynaptic release of acetylcholine and binding to postsynaptic receptors (Grill et al. 2011). Ultimately, this results in peripheral neuropathology.

1.6 Cholesterol

The cholesterol biosynthetic pathway is integral to proper cellular functioning and is notably disrupted in several neurodegenerative disease processes (Petro et al. 2016). The chemical structure of cholesterol includes 27 carbons, a hydrocarbon tail, a central sterol nucleus made of four hydrocarbon rings, and a hydroxyl group. Common to all steroid hormones, which are ultimately derived from cholesterol, is the center sterol nucleus or ring. Notably, the hydrocarbon tail and the central ring are both non-polar structures and therefore cannot be carried in the blood alone. Consequently cholesterol, also known as a lipid, is packaged together with proteins called apoproteins that then allow it to become polar enough to travel through the blood circulation as what is known as a lipoprotein (Craig et al. 2021). These lipoprotein structures are most recognizable as

high-density lipoprotein (HDL) and low-density lipoprotein (LDL), both of which are commonly measured for heart health by primary care physicians.

Cholesterol is obtained by the body in two main ways, (1) from the diet and (2) it can be synthesized *de novo*, i.e., from new. Due to its bulky size, hydrophobic tail, and overall non-polar characteristics, cholesterol cannot cross the blood brain barrier. Therefore, the brain must produce its own cholesterol separately from the body's regular metabolic processes. Due to this, cholesterol is not taken up by the blood as with peripheral tissues and is made *de novo* in the brain. The brain is the most cholesterol-rich organ and contains about 20% of the body's cholesterol (Björkhem et al. 2004). As previously mentioned, cholesterol is an important substituent of the myelin sheath in the axons of neurons and makes up about 70% of its presence in the CNS while the remaining 30% can be found in plasma and subcellular membranes of neurons and astrocytes (Arenas et al. 2017). It has many functions in addition to being an important structural component for cellular membranes and myelin, it is also a precursor of steroid hormones and bile acid synthesis as well as integral to synapse and dendrite formation (Gortiz et al. 2005 and Fester et al. 2009). Cholesterol biosynthesis begins from the molecule acetyl-CoA. Acetyl-CoA is first converted to 3-hydroxy-3-methylglutaryl-CoA by the enzyme HMG-CoA. 3-hydroxy-3-methylglutaryl-CoA is then converted to mevalonate by HMG-CoA reductase (HMGCR). Notably, HMG-CoA is known as the rate-limiting and irreversible step in cholesterol synthesis. Next, a series of enzymatic reactions occur converting mevalonate into 3-isopentenyl pyrophosphate, farnesyl pyrophosphate (FPP), squalene, lanosterol, and additional 19-step progression until the final product, cholesterol (Berg 2002). Neurons and astrocytes play complementary roles in the

cholesterol biosynthetic pathway. In astrocytes, you will largely find the product cholesterol whereas in neurons lanosterol is more widely found. Despite this, astrocytes have a higher presence of lanosterol 14-α-demethylase (CYP51), a lanosterol converting enzyme (Zhang et al. 2015). This demonstrates neuron's poor conversion abilities and establishes the need for astrocytes to help regulate neuronal cholesterol. During cholesterol efflux, enzymes such as cholesterol 24-hydroxylase (CYP46A1; Lange et al. 1995) are responsible for hydroxylating cholesterol to 24-hydroxycholesterol (24-OHC). Furthermore, expression of CYP46A1 is low in glial cells and this eludes to neurons being the major site of cholesterol turnover in the brain (Ramirez et al. 2008). In order to maintain proper levels of cholesterol, cells employ sterol regulatory element-binding proteins (SREBPs) which are transcription factors that regulate the transcription of genes that encode for enzymes to increase or decrease cholesterol (Brown et al. 1999). Two specific enzymes known to be upregulated are CYP51 and squalene synthase (farnesyl diphosphate farnesyl transferase 1; fdft1), which converts FPP to squalene. In contrast, these enzymes can be downregulated by nuclear receptors such as liver x receptors (LXRs; Wang et al. 2008). LXRs also mediate cholesterol efflux by regulating proteins such as APOE, ABCA1, and APOA1 (Pfrieger et al 2011). APOE and APOA1 are both apolipoproteins and ABCA1 is an ATP-binding cassette (ABC) transporter but both works together in transporting cholesterol in the brain (Zhang et al. 2015). HMGCR and APOE can both be used as upstream and downstream products, respectively, in the cholesterol biosynthesis pathway and can give indications for dysregulation (Nunes et al. 2013).

1.7 Neurodegeneration

Neurotoxic outcomes can greatly resemble neurodegenerative processes. The two are often intricately linked. Neurodegeneration describes a progressive loss of structure, function, or death of neurons. The hallmarks of neurodegeneration are oxidative stress, proteasomal impairment, mitochondrial dysfunction, and the accumulation of protein aggregates (Tan et al. 2015). Although aging is the number one cause of neurodegeneration, there is increasing research being done on ways to minimize or slow this process and potentially avoid some of the more severe symptomologies.

The classification of neurodegenerative diseases can be quite complicated with a lot of signs and symptoms overlapping. However, generally, they are classified by their most predominant feature or lesion. For example, CNS disorders can be initially grouped by diseases that present in the cerebral cortex, basal ganglia, brainstem, and cerebellum, or the spinal cord (Przedborski et al. 2003). Diseases that predominantly involve the basal ganglia are essentially characterized by abnormal movements, Parkinson's disease (PD) is an example of this and demonstrates a lack of voluntary movements (Stacy et al. 1992). Furthermore, diseases of the cerebellum can present as dentatorubral degeneration, where the most conspicuous lesions are in the dentate and red nuclei. Additionally, Machado-Joseph disease where degeneration comprises the lower and upper motor neurons, the substantia nigra, and the dentate system (Przedborski et al. 2003). Spinal cord neurodegenerative diseases include amyotrophic lateral sclerosis (ALS) that details severe lesions found in the anterior part of the spinal cord. And Friedreich ataxia, where severe lesions are found in the posterior part of the spinal cord (Przedborski et al. 2003).

Clinically, neurodegeneration manifests itself as resting tremor, ataxia, dementia, mood changes, and more. In a mimicry fashion, molecular signs of neurodegeneration

follow clinical signs and present in several ways. For Alzheimer's Disease (AD) one could find plasma/CSF $A\beta_{1-42}$ peptide, tau protein in CSF, and phosphor-tau. For PD, loss of dopamine transporters and Lewy bodies. For ALS, mGLUR2, SOD1, Glutathione, 8OH2'dG, and cytokines. And for Huntington's Disease (HD), growth hormones, cytokines, mGLUR2, SOD1, glutathione, and 8OH2'dG. (Agrawal et al. 2015). These are signs that can be validated through PET, magnetic resonance imaging (MRI), and nuclear magnetic resonance spectroscopy (NMRS). Ultimately, clinical and molecular symptomology mirror each other in generally predictable patterns that allow researchers to better predict molecular and clinical-pathological outcomes.

Similarly, genetics can play a large role in pathological outcomes as well. As previously mentioned with cytochrome P450 enzymes, genes can also code for increased or decreased enzymatic functions that determine the physiological outcomes of diseases. During neurodegeneration, some genetic diagnostic markers for AD are amyloid precursor protein mutations, presenilin-1/2 gene mutations, and ApoE isoforms and polymorphisms. For PD, α-synuclein, parkin, UCH-L1, PINK1, DJ-1, and NR4A2 gene mutations. For ALS, ALS2, NEFH, SOD1, C0orf72, FUS, and TARDBP gene mutations. And finally, for HD, HTT gene mutations (Agrawal et al. 2015).

Aside from the major hallmarks of neurodegeneration, another important component involved in these pathologies is cholesterol. Various cholesterol markers such as APOE and CYP46A1 are dysregulated in diseases such as AD (Mahley et al. 2006 and Arenas et al. 2017). Overall cholesterol levels are increased in AD patients (Cutler et al. 2004). Primary neuronal cell culture studies have even shown an overload of

cholesterol at the plasma membrane leading to an increase in Aβ production (Marquer et al. 2011).

Drugs and specifically, neurotoxins, can contribute to neurodegenerative outcomes. Neurotoxicity could be even be thought of as neurodegeneration specifically caused by a drug. However, the line for that is thin as any disease can have multifactorial etiologies. LPS is an example of this and has been cited as a model for neuroinflammation that is associated with neurodegeneration (Batista et al. 2019). Nevertheless, neurodegeneration is important to scientists because the brain/nervous system is the driving force for human function. Furthermore, aging is a disease we all succumb to and therefore makes pathologies of the brain relevant to every living organism.

1.8 Knowledge gaps

Highly funded research can be found exploring etiologies, pathologies, therapeutics, diagnostics, and more surrounding neurodegeneration. There is an increasing desire to better understand the process of neurodegeneration as well as therapeutic options for different diseases. Due to neurodegeneration and neurotoxicity sharing many similarities, it is also of interest to investigate drug causatives that share similar disease pathologies.

Furthermore, the use of animal models in neurodegenerative and neurotoxicological research can be very limited. Few, if any, animals can be considered suitable to accurately model neurodegeneration. Notably, mice, although a very popular animal in research laboratories, do not naturally develop neurodegeneration like humans do. Due to this, most studies take advantage of the use of various biomedical technologies to “infect” mice with neurodegenerative-like pathologies. In addition to this, many ethical

concerns can arise with the use of animal models in such pathological research leading many research labs to move towards more humane substitutions. One such substitution is computational modeling. Also known as, *in silico*, computer simulated modeling can be advantageous over animals due to costs, specificity, efficiency, and several other reasons. Previously, I gave very brief summations of brain anatomy, neurons, glia, cholesterol, antibiotics, the biotransformation of drugs, neurotoxicity, and neurodegeneration. With this knowledge, as you will see in the latter body of this work, we were able to combine and apply it to create several computer-generated physiologically based pharmacokinetic models of human, horse, mouse, and rabbit species in order to simulate and predict neurotoxicological outcomes in the brain given a drug.

Here we aim to investigate if vulnerable populations of humans and animals to metronidazole neurotoxicity demonstrate dysfunction of glial cholesterol metabolism.

Chapter 2

Antibiotic neurotoxicity and links to neuroinflammation

Introduction

2.1 Background

Metronidazole is a broad-spectrum antibiotic prescribed in human and veterinary medicine to treat a variety of diseases including pelvic inflammatory disease, endocarditis, bacterial vaginosis, dracunculiasis, giardiasis, trichomoniasis, and amebiasis (Baggot 1988). It belongs to a class of drugs called nitroimidazoles known to combat anaerobic bacterial and protozoal infections (Sweeney et al. 1991). Reported use of metronidazole can be seen in humans, horses, dogs, cats, rabbits, fish, reptiles, as well as many others (Kuriyama et al. 2011, Sweeney et al. 1991, Tauro et al. 2018, Evans et al. 2003, Caylor et al. 2001, Jokipii et al. 1977, and Innis et al. 2007). Furthermore, metronidazole is also listed as one of the World Health Organization's (WHO) most essential medicines (WHO 2015). Due to its efficiency and cost-effectiveness, it comes as no surprise that it is utilized as a staple across several different species (Kuriyama et al. 2011). It has a half-life of 8 hours and is mainly metabolized via hydroxylation in humans (other species, like mice, mainly use glucuronidation) via the liver by an enzyme called CYP2A6. After administration, less than 20% of it binds to plasma proteins and it is largely excreted in the kidney, mainly as the hydroxylated metabolite and minimally as the parent compound. With repeated dosing, small accumulations can occur. Further, it readily crosses the blood-brain barrier as well as other tissues (i.e., placenta) (Bucklin 2014). The toxicological profile is as follows: Oral LD50 (rat): 3000 mg/kg; Oral LD50 (mouse): 3800 mg/kg; Intraperitoneal LD50 (mouse): 870 mg/kg; Subcutaneous LD50

(mouse): 3640 mg/kg; Oral TDLO woman 12 mg/kg; Oral TDLO (man): 3.57 mg/kg; TDLO = lowest published toxic dose (Cavalleri et al. 1977, Konopacka et al.1990, Kirkham et al. 1986, Fagin 1965).

While many documented side effects of metronidazole are reasonably benign, several human and veterinary cases of central nervous system toxicity have been reported, leading to rare instances of seizures and severe ataxia. Incidences of this neurotoxicity amount to less than 1% of human cases and were cited as occurring in both peripheral and central nervous systems (Guglielmo 2020). Most often, these effects are reversible with discontinued use. However, the mechanistic information surrounding this neurotoxicological outcome is largely unknown. Proposed mechanisms include axonal swelling with increased water content due to toxic injury, vascular spasm with mild reversible localized ischemia, modulation of the gamma-aminobutyric acid receptors within cerebellar and vestibular systems, and RNA binding with inhibition of protein synthesis and axonal degeneration (Cação et al. 2015, Iqbal et al. 2013, Kim et al. 2011). Due to the inconsistencies of the possible causes of neurotoxicity, there is a need for more predictive quantitative studies. Although metronidazole has been a drug of choice for decades, the mechanism of this marked phenomenon has yet to be determined and is poorly researched.

Current literature has neither concluded a mechanism of action of this unintended neurotoxicity nor established appropriate mammalian dosages to avoid this variably adverse outcome. In previous reports, the drug has been found to be neurotoxic at varying dosages and thus been cited as a non-dose-related phenomenon (Kuriyama et al. 2011). For our work, we utilize predictive simulations that mainly focus on dose-related

mechanisms of toxicity. Despite there being no recognized mechanism, related themes in clinical neurotoxic reports mention axonal degeneration, especially in humans (Cação 2015, Wexler 2014). Furthermore, Xiao et al. performed a cell viability experiment revealing that metronidazole treated primary and neuronal cell lines induced apoptotic cell signaling, however not in a reactive oxygen species (ROS) dependent manner. The authors went on to mention that this was in line with the properties of metronidazole being bacteriostatic rather than bactericidal and favoring an inhibition of metabolic pathways (Xiao et al. 2018).

Metronidazole's ability to cross the blood-brain barrier makes it a cause for concern in its rare unintended neurotoxic effects (Frasca 2013). Kuriyama et al. did a clinical case review of metronidazole neurotoxicity stating that these effects generally subside with discontinued use (Kuriyama et al. 2011). Correspondingly, if metronidazole favors a bacteriostatic functionality as indicated above, we believe that this reversible phenomenon is highly plausible. At the lowest cumulative dosages, acute mental status change was reported in 21 of 64 patients. In these cases, acute mental status change referred to confusion and/or disorientation. This may lead to the belief that there is a possibility that many other human patients could be experiencing some sort of mild neural-modulatory effects from taking metronidazole without the knowledge to report or document it. Additionally, in several of the case reports of human and canine toxicities, the patients were documented as being prescribed metronidazole for various inflammatory-based illnesses such as endocarditis, enteritis, chronic diarrhea, Chron's disease, colitis, etc. (Tauro et al. 2018 and Kuriyama et al. 2011). This gives us reason

to believe that localized and/or systemic inflammation is the determining factor for vulnerability to this drug.

2.2 Cytochrome P450 51A1

Cholesterol is a major component of neuronal cells. Largely residing within the axon, the myelin membranes are comprised of cholesterol and essential for insulation, transport, and communication between neurons. Although a majority of cholesterol is synthesized by oligodendrocytes, astrocytes play a very large role in cholesterol biosynthesis in the brain. Ferris et al. demonstrated that knockdown of SREBP2 in the astrocytes of mice demonstrated impaired brain development and behavior deficits (Ferris et al. 2017). This demonstrates how essential glial cells are for proper neuronal function. All of this is critical for higher-order animals because neurons rely on this proper insulation for accurate and fast transduction of their signals. Multiple neurodegenerative diseases have even been linked to poor myelination etiologies (Saher 2015).

As an azole, metronidazole, is a part of a class of compounds known for their antifungal effects. Azoles have a function known to be inhibitory of CYP51A1, sterol 14α-demethylase (CYP51). CYP51 is within the family of Cytochrome P450s recognized for their role in biochemical transformations. It is a highly regarded enzyme for its conserved homology across kingdoms of life (Kelly 2001). Furthermore, in mammals, CYP51 is involved in cholesterol biosynthesis in both the central and peripheral nervous systems (Saher 2015). It is a key component for the *de novo* synthesis of cholesterol that is crucial for neuronal function.

2.3 Reductive characteristics

Literature has highlighted a unique characteristic about this drug, namely, the ability of the nitro group on its imidazole ring to be reduced. This reduction is its main

mechanism of action in bacteria. Specifically, in *Bacteroides fragilis* it induced rapid inhibition of DNA replication where the DNA remained intact, DNA polymerase was unaffected, and RNA and protein remained synthesized at similar rates (Sigeti 1983). Acting as an alternate electron acceptor, this reduction phenomenon has been cited to produce a reactive metabolite that can bind and/or fragment DNA (Dingsdag 2018). In that article, the authors proposed the reduced metabolite to form a radical anion at the nitro group and for this to be detoxified into 5-amino, N-(2-hydroxyethyl)-oxamic acid, and acetamide metabolites (Dingsdag 2018). This ability of metronidazole to be reduced differentiates it from other azoles in its class. While other azoles also have the ability to cross the blood-brain barrier and have also been reported to cause neurotoxicity, these cases seem to be largely dose-related. The ability of metronidazole to be reduced to a reactive metabolite may provide insight as to specifically why low doses of this drug are capable of causing neurotoxicity.

In summation, we believe vulnerable populations of humans and animals to be experiencing inflammatory distress that subsequently reduces metronidazole to a toxic metabolite capable of inhibiting CYP51 and disrupting glial cholesterol metabolism to ultimately reveal a neurotoxic phenotype. Due to this anti-fungal action of inhibiting a highly conserved protein within the cholesterol biosynthesis pathway, our aim here is to investigate if a portion of this belief holds true in murine glial cells treated with and without LPS. Thereby we aim to better understand vulnerable populations of humans and animals as well as take a first look into a more comprehensive elucidation of the mechanism behind these neurotoxicities.

Materials and Methods

2.4 Simulation

AnyLogic, a multimethod simulation program, was used to couple a pharmacokinetic model of metronidazole dosimetry with a cellular response model of theoretical neurological dysfunction. Physiologically based pharmacokinetic models for the human, horse, mouse, and rabbit were built on this platform utilizing experimental physiological parameters for each respective species. Fractions of body weights, body volumes, and tissue blood volumes were multiplied by their respective values to achieve a functional model (see Table 1).

Body volume was calculated for the mouse and rabbit via an equation taken from Utturkar et al.:

$$Bodyvolume = \frac{Bloodvolume}{Bloodvolumefraction}$$

Additionally, the cardiac output for the mouse and rabbit was also calculated from Utturkar et al.:

$$CardiacOutput\left(\frac{L}{min}\right) = 0.275(BW)^{0.75}$$

In order to validate our computational simulation model blood plasma and cerebral spinal fluid (CSF) concentrations were obtained from human, equine, murine, and rabbit experimental data. Frasca et al. obtained pharmacokinetic parameter values of metronidazole distributed in four human patient's CSF through intravenous infusions of 500 mg over 0.5 hours every 8 hours (Frasca 2014). Bergamaschi et al. obtained human plasma pharmacokinetic values of metronidazole through a single 750 mg oral dose with

13 volunteers (Bergamaschi 2013). Pharmacokinetic CSF values of metronidazole for the horse were obtained from Specht et al. with a specific dosing scheme of one loading dose of 15 mg/kg and then 4 maintenance doses of 7.5 mg/kg over a period of 24 hours. Mean CSF was taken 3 hours following the fourth maintenance dose, at 4.3 µg/ml (Specht 1992). Horse plasma concentrations were obtained by Baggot et al. with a 250 mg dose of metronidazole administered via a nasogastric tube (Baggot 1998).

All experimental data points were recorded and entered into the simulation software to be validated and provide reproducible results (Figure 1, 2, 3, and 4). Mouse experimental data was collected after a 500 mg/kg oral dose of metronidazole was given. Pharmacokinetic values were taken over 12 hours for the CSF and plasma (Chew 2012). Brain concentrations were taken in µg/g and converted to µg/ml to reflect the other models. Rabbit pharmacokinetic values were obtained after a 50 mg intravenous injection of metronidazole into a lateral ear vein (Jokipii 1977). For the simulation, this dose was modeled orally without issue, despite being an IV injection, and plasma and CSF values were able to be appropriately validated.

Values for physiologically based pharmacokinetic models for the human, horse, mouse, and rabbit biological models were built using parameters from Hoffman et al., Webb et al., and International Life Sciences Institute (ILSI). Rabbit brain blood flow values were more difficult to find and therefore found separately with values taken from Fischer et al. and then multiplied by the same respectively calculated cardiac output.

In order to establish a simulated threshold dose of neurotoxic effects, data were pulled from Xiao et al. A cell survival study was done on various neuronal cell lines treated with metronidazole. Significance among all groups was identified as the minimum

concentration for neuronal dysfunction for our work (Xiao et al. 2018). This value was 20 µg/ml converted to 20 mg/L.

Table 1: PBPK model parameters. *Parameters of the physiologically based pharmacokinetic models as they are seen within human, equine, mouse, and rabbit species.*

Physiological Parameters	Symbol	Human Values[a]	Equine Values[b]	Mouse Values[c,d]	Rabbit Values[d]
Bodyweight (kg)	BW	60	380	0.025	3
Body volume (L)	BV	-	-	0.0326	2.773[c]
Tissue Volumes (fraction of BW or BV)					
Fraction of liver volume	LV	0.026	0.0143	0.055	0.0308
Fraction of HP tissue volume	HPV	0.25-CNSV-LV 0.25-0.02-0.026	0.25-CNSV-LV[a] 0.25-0.002-0.0143	Lung + Heart + Kidney 0.007, 0.05, 0.017	Lung + Heart + Kidney 0.0072, 0.002, 0.0052
Fraction of PP tissue volume	PPV	1-Sum	1-Sum	1-Sum	1-Sum
Fraction of brain volume	CNSV	0.02	0.002	0.017	0.0056
Fraction of blood volume	BLV	0.079	0.09	0.0613	0.066
*Chemical- and Tissue-Specific Partition Coefficients**					
Liver to blood partition coefficient (BA/HA)	KL	1	1	110.607	0.1
HP tissue to blood partition coefficient (BA/HA)	KHP	1	1	1	1
PP tissue to blood partition coefficient (BA/HA)	KPP	1	1	1	1
Brain to blood partition coefficient (BA/HA)	KCNS	1.763	0.265	0.386	0.666
Elimination constant	KElim	3.659	23.560	85.275	2.140
Tissue Blood Flows (fraction of Q)					
Cardiac output (L h_{-1})	Q	322.8	1,367.5	1.0374	37.6
Fraction of liver blood flow	QL	0.25	0.034	0.161	0.334
Fraction of highly perfused blood flow	QHP	1-Sum	1-Sum	1-Sum	1-Sum
Fraction of poorly perfused blood flow	QPP	0.19	0.19	Pancreas + Bone + Fat + Uterus 0.0157, 0.0407, 0.036, 0.0087	Muscle + Adipose 0.01698, 0.0604
Fraction of brain blood flow	QCNS	0.114	0.047	0.033	0.0287

[a]Hoffman et al.
[b]Webb et al.
[c]Utturkar et al.
[d]ILSI
*Calibrated values

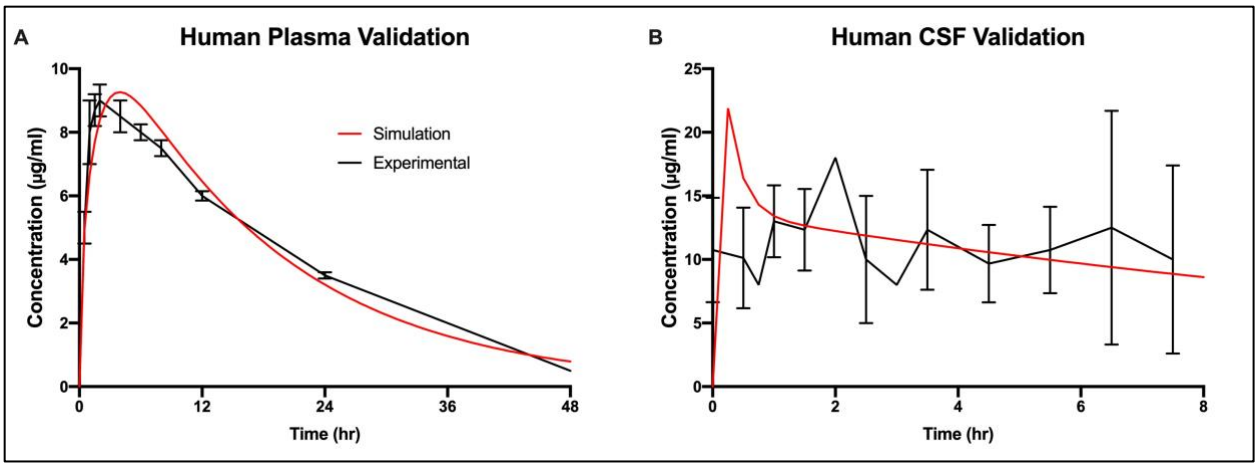

Figure 1: Computationally simulated data is analogous to literature concentration of metronidazole in the plasma and CSF of human. *(A) Overlay of experimental (Bergamaschi 2013) and simulation human metronidazole plasma data. (B) Overlay of experimental (Frasca 2014) and simulation human metronidazole cerebrospinal fluid data.*

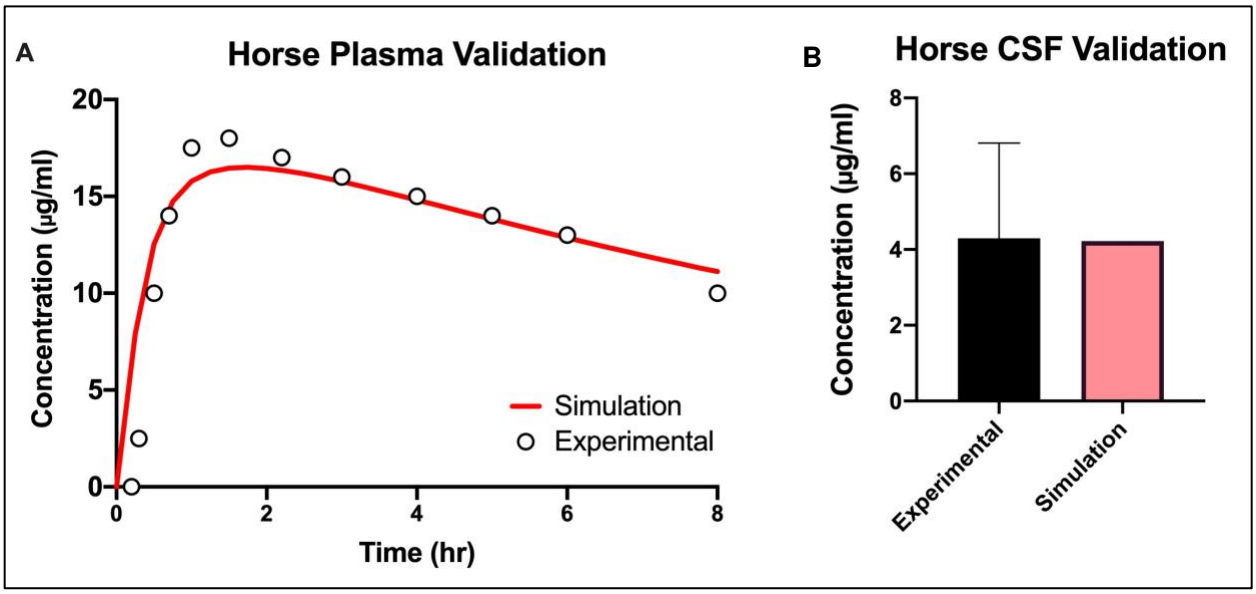

Figure 2: Computationally simulated data is analogous to literature concentration of metronidazole in the plasma and CSF of horse. *(A) Overlay of experimental (Baggot 1988) and simulation horse metronidazole plasma data (B) Comparison of mean-derived cerebrospinal fluid concentrations of metronidazole in experimental (Secht 1992) and simulation data.*

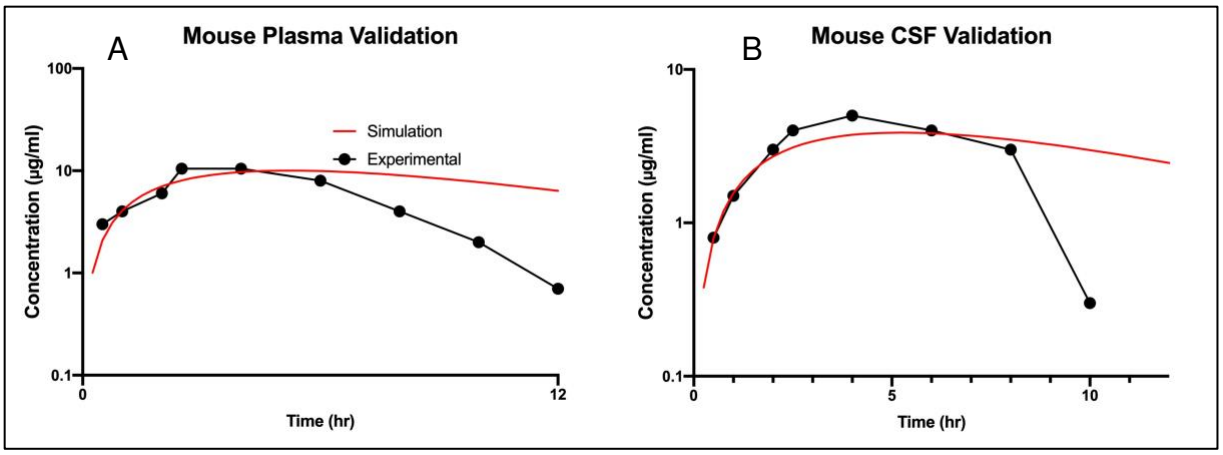

Figure 3: Computationally simulated data is analogous to literature concentration of metronidazole in the plasma and CSF of mice. *(A) Overlay of experimental (Chew 2012) and simulation mouse metronidazole plasma data. (B) Overlay of experimental (Chew 2012) and simulation mouse metronidazole cerebrospinal fluid data.*

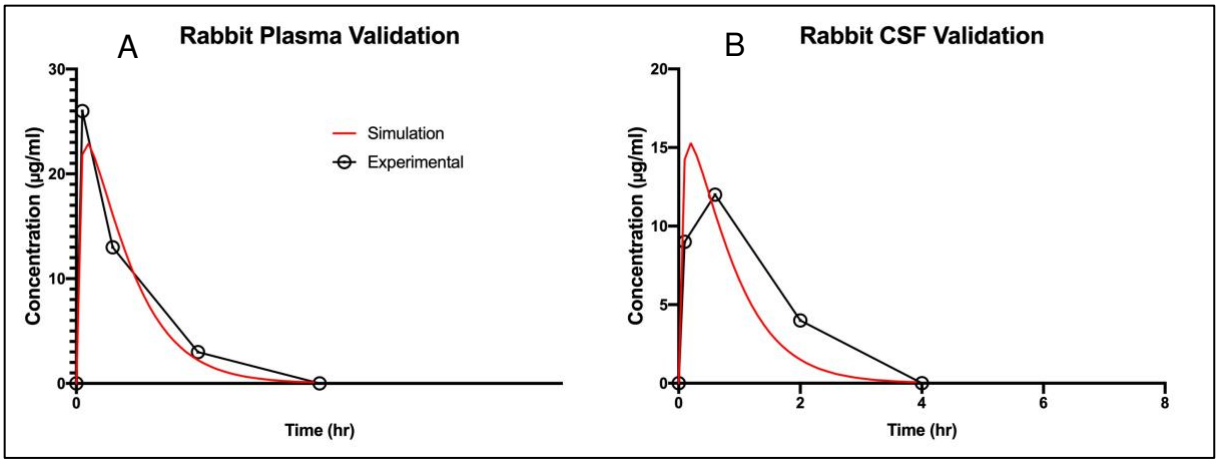

Figure 4: Computationally simulated data is analogous to literature concentration of metronidazole in the plasma and CSF of rabbit. *(A) Overlay of experimental (*Jokipii 1977*) and simulation rabbit metronidazole plasma data. (B) Overlay of experimental (*Jokipii 1977*) and simulation rabbit metronidazole cerebrospinal fluid data.*

2.5 Isolation of primary glial cells and cell culture

A C57 primary murine mixed cerebellar astrocyte and microglial culture were used for all cellular assays. Cells were harvested either on or between post-natal days 3-5 and isolated as previously described (Kirkley et al. 2017) from both female and male rodents. Media consisted of MEM/EBSS supplemented with 10% fetal bovine serum (FBS) and 5% Penicillin/Streptomycin.

2.6 Cell viability

Primary glial cell death was measured with the Vybrant® MTT Cell Proliferation Assay Kit. Glial cells were plated in a 96-well and then spiked with respective metronidazole treatments (10 µg/ml, 20 µg/ml, 30 µg/ml, 40 µg/ml, 50 µg/ml, and 60 µg/ml) for 72 hours. For vulnerability experiments, the media was replaced with 500 ng/ml or 1 µg/ml concentrations of LPS for 24 hours and then spiked with metronidazole for 72 hours. The total time for LPS treatment was 96 hours. The assay was done in technical duplicates with results of duplicate biological experiments. Optical density was read on a VersaMax with emission detection at 540 nm.

2.7 RT-PCR

iQ™SYBR® Green Supermix (Bio Rad) was used for real-time polymerase chain reaction(RT-PCR) analysis for 3-hydroxy-3-methylglutaryl-coenzyme A reductase (HMGCR), lanosterol 14-α-demethylase (CYP51), and apolipoprotein E (APOE). iScript™ Reverse Transcription Supermix for RT-qPCR (Bio Rad) was used for reverse transcriptase. Primers were made as previously described for APOE and HMGCR (Nunes et al. 2013). The assay was done in technical duplicates with results from a single experiment.

2.8 Cholesterol synthesis

Cholesterol synthesis was measured using the AMPLEX® Red Cholesterol Assay Kit from ThermoFisher Scientific following the manufacturer's instructions. Glial samples were plated and treated with respective metronidazole and LPS concentrations in 6-well plates for their predetermined time periods. Samples were then collected from the plates and resuspended in the 1X reaction buffer provided in the kit. Immediately following collection, the samples were vortexed and sat on ice for 30 minutes. The samples were then plated in a 96-well plate and cholesterol was normalized by loading each well with equal amounts of protein (10 µg). After initiating the reaction by adding 50 µl of the AMPLEX® Red reagent/HRP/cholesterol oxidase/cholesterol esterase, the plate was incubated for 30 minutes at 37º C. Each assay was in duplicate and the results were obtained from at least 2 individual experiments. Optical density was read on a VersaMax with emission detection at 570-600 nm.

2.9 Liquid chromatography/Mass spectrometry

To confirm the presence of metronidazole LC/MS was used to validate. Metronidazole was solubilized in deionized water at room temperature. Figure 5 illustrates the substantiation of the parent compound, metronidazole, via its molecular weight. The molecular weight of metronidazole is 171.15 g/mol and from there we add ~1 g/mol to account for the protonation of the compound once through the mass spectrometer. Therefore, the expected weight for metronidazole is ~172.1 g/mol. For the reduction experiments, metronidazole was solubilized in MEM/EBSS (supplemented with 10% FBS and 5% Pen/Strep) and sodium dithionite at 37º C. After 1 hour the sample was analyzed.

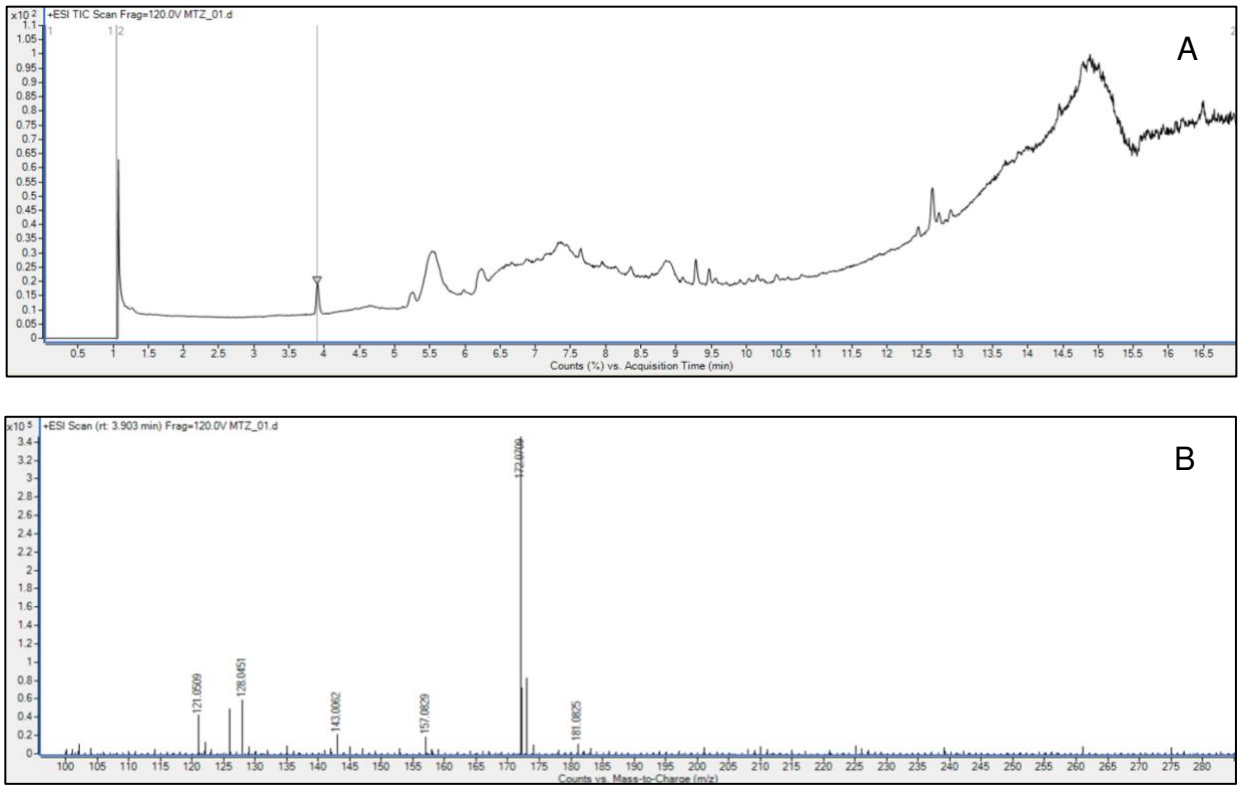

Figure 5: LC/MS identifies molecular weight of metronidazole. *(A) The top panel illustrates the peak of interest, metronidazole, coming off the liquid chromatography column around the 4-minute mark. All of the peaks 5 minutes and beyond are considered unimportant extraneous molecules. (B) In the bottom panel the peak of interest from the top panel is further analyzed and the compound of interest is demonstrated by the tallest line and verified by its molecular weight at 172.0709 g/mol. The smaller lines are considered nonsignificant.*

Results

2.10 Simulated dosing schemes

Human CNS concentrations after oral tablet doses of 250 mg, 500 mg, 750 mg, 1000 mg, or 1500 mg of metronidazole were simulated using a repeated dosing scheme of 4.2 mg/kg, 8.3 mg/kg, 12.5 mg/kg, 16.6 mg/kg, and 25 mg/kg, respectively (Figure 6). A line was drawn at 20 mg/L to indicate nitroimidazole neuronal dysfunction. The repeated dosing scheme followed 120 hours with a dose applied every 12 hours. Typically prescribed therapeutic doses are 4.1 mg/kg and 8.3 mg/kg which roughly translates to a 250 mg and 500 mg oral tablet based on a 60 kg human model, respectively. The concentration is 80 mg/L indicating higher and more prolonged concentrations of metronidazole that stay in the cerebral spinal fluid of humans.

Horse CNS concentrations were simulated as a repeated dosing scheme after an oral dose of 7.5 mg/kg, 15 mg/kg, 22.5 mg/kg, or 30 mg/kg of metronidazole (Figure 7). Concentrations were monitored over 120 hours with a dose applied every 6 hours. Typically prescribed therapeutic doses are 15 mg/kg for the horse. The concentrations go up to ~22 mg/L indicating lower concentrations of metronidazole that stay in the cerebral spinal fluid of horses.

Mouse CNS concentrations were simulated after therapeutic doses of 25 mg/kg, 20 mg/kg, 7.5 mg/kg, 3 mg/kg (Figure 8). Typically prescribed therapeutic doses are 15 mg/kg for the mouse. The Y-axis goes up to 25 mg/L, however, the concentrations of metronidazole stay well below 0.5 mg/L indicative of their high tolerance for the drug.

Rabbit CNS concentrations were simulated at oral doses of 25 mg/kg, 20 mg/kg, 15 mg/kg, 10 mg/kg (Figure 9). Typically prescribed therapeutic doses are 20 mg/kg for

the rabbit. The Y-axis goes up to 25 mg/L and the repeated dosing scheme demonstrates a rapid clearance of the drug.

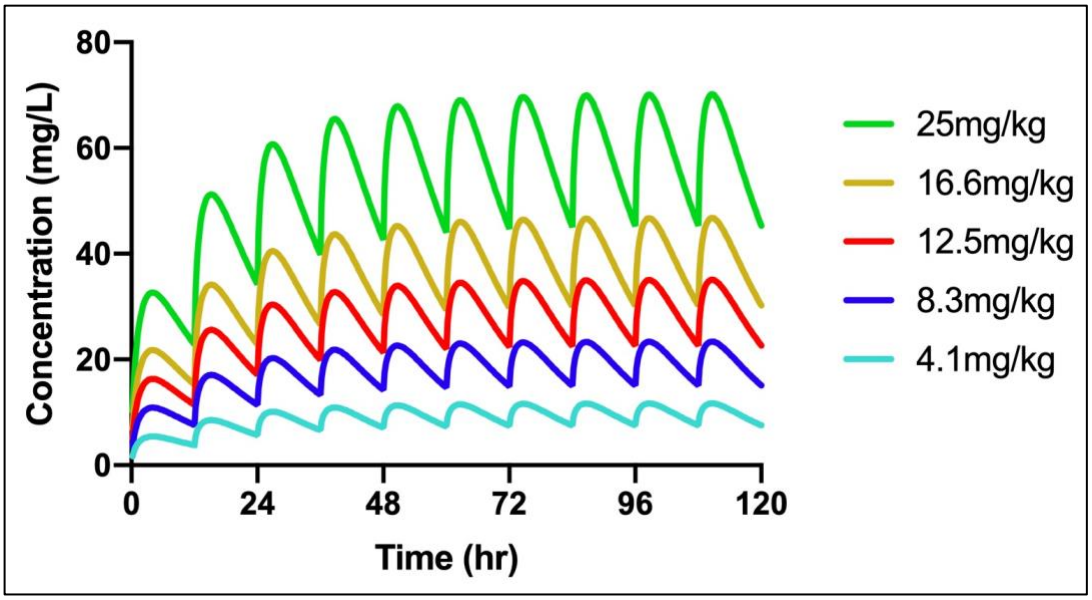

Figure 6: Predicted simulation of central nervous system concentrations of metronidazole in human*. Drug given as an oral tablet given every 12 hours (hr) in a human model.*

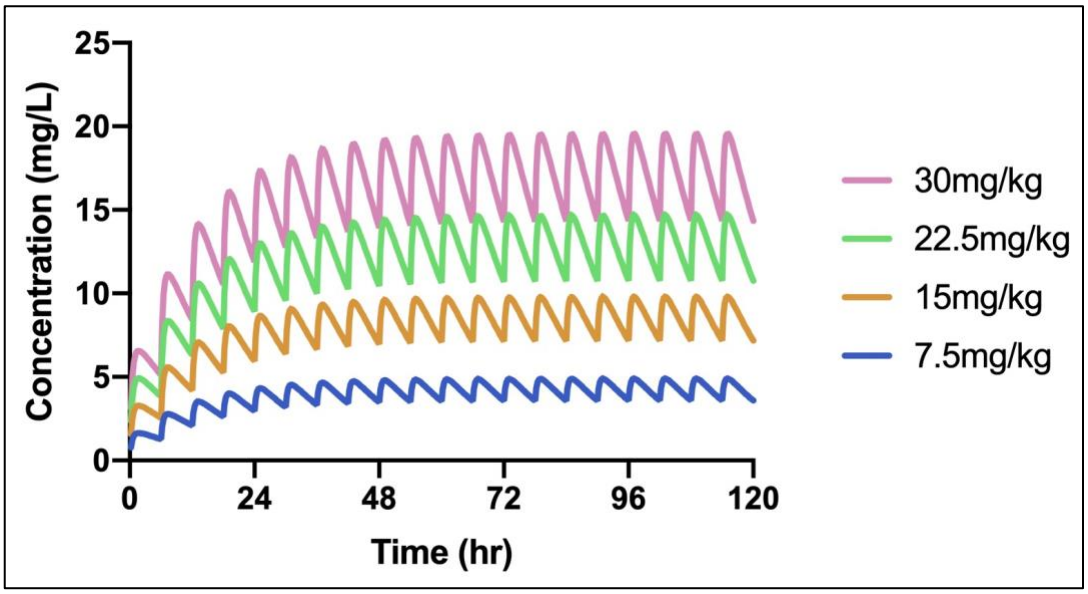

Figure 7: Predicted simulation of central nervous system concentrations of metronidazole in horse. *Drug given as an oral tablet given every 6 hours in an equine model.*

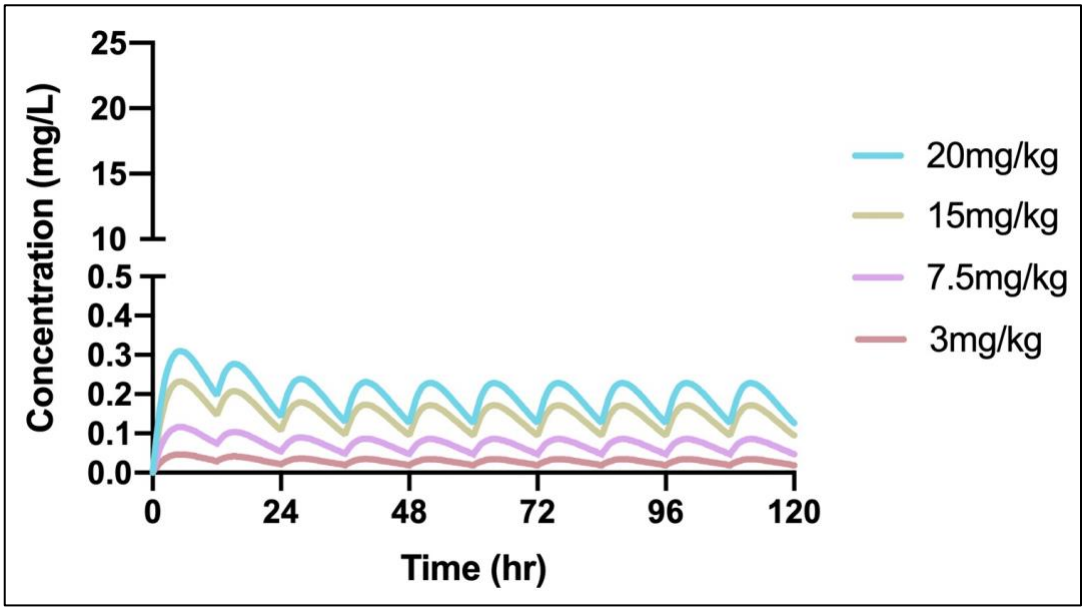

Figure 8: Predicted simulation of central nervous system concentrations of metronidazole in mouse. *Drug given as an oral tablet given every 12 hours in a murine model.*

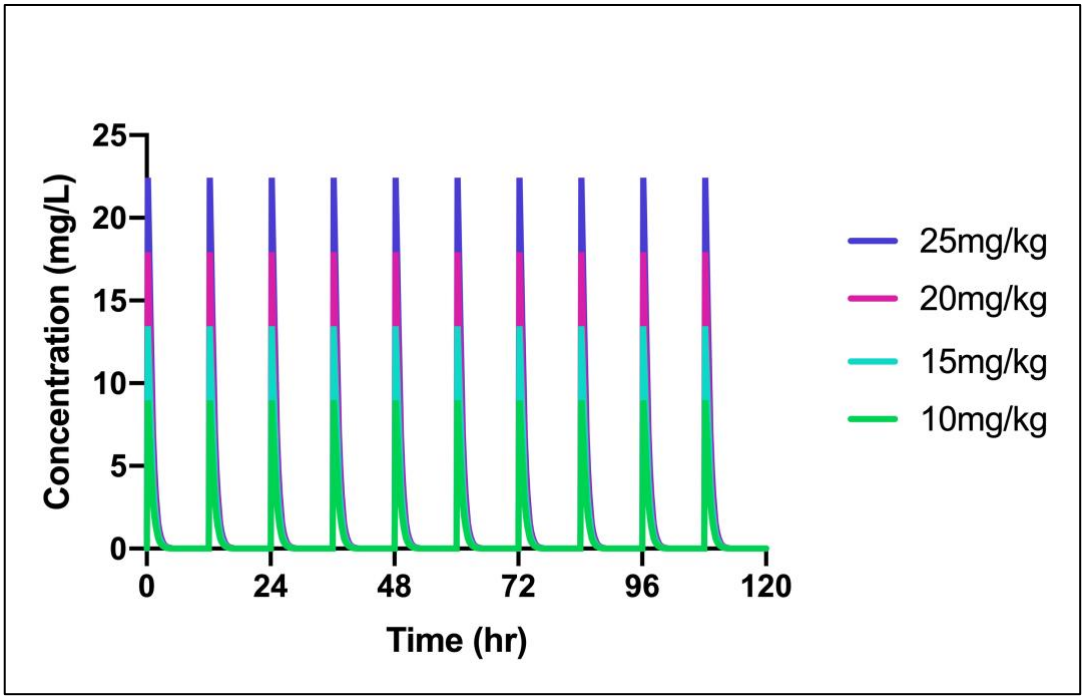

Figure 9: Predicted simulation of central nervous system concentrations of metronidazole in rabbit. *Drug given as an oral tablet given every 12 hours in a rabbit model.*

2.11 Simulated neuronal dysfunction

Typically prescribed therapeutic doses for humans are 4.1 mg/kg and 8.3 mg/kg which roughly translates to a 250 mg and 500 mg oral tablet based on a 60 kg human model, respectively. In Figure 10 the concentration goes up to 80 mg/L indicating higher and more prolonged concentrations of metronidazole that stay in the cerebral spinal fluid of humans well above the predicted neurotoxicological threshold.

Normally prescribed therapeutic doses are 15 mg/kg for the horse. The Y-axis goes up to 25 mg/L indicating lower concentrations of metronidazole that stay in the cerebral spinal fluid of horses and stay below the predicted neurotoxicological threshold.

Commonly prescribed therapeutic doses are 15 mg/kg for the mouse. The graph concentration goes up to 25 mg/L, however, the predicted concentrations of metronidazole stay well below 0.5 mg/L indicative of their high tolerance for the drug.

Characteristically prescribed therapeutic doses are 20 mg/kg for the rabbit. The Y-axis goes up to 25 mg/L and the repeated dosing scheme demonstrates a rapid clearance of the drug and is still well within the predicted neurotoxicological threshold.

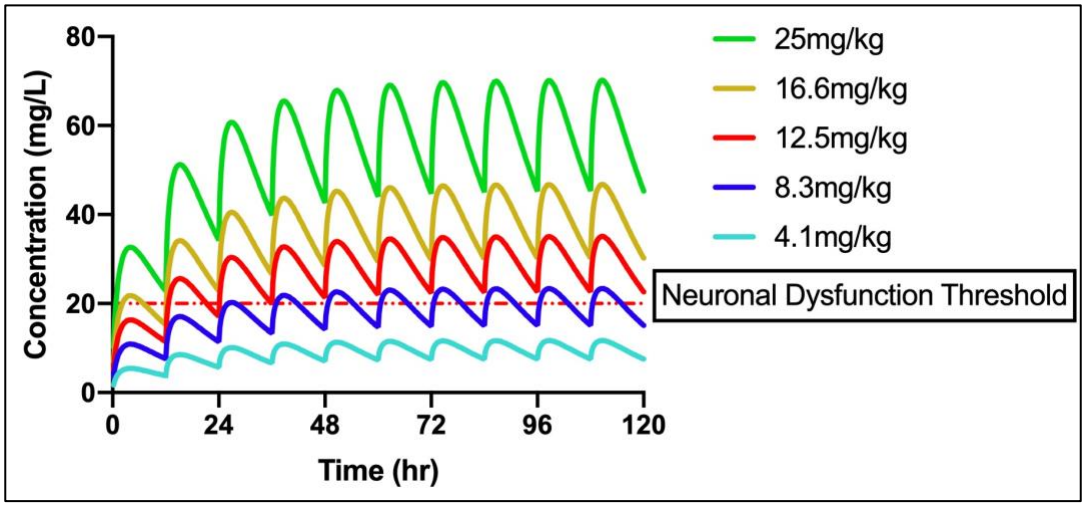

Figure 10: Computationally predicted neurotoxicity in human model. *A neurotoxicological threshold is applied at 20 mg/L of the predicted simulation of central nervous system concentrations of metronidazole. The graph represents a repeated dosing scheme of an oral tablet given every 12 hours in a human model.*

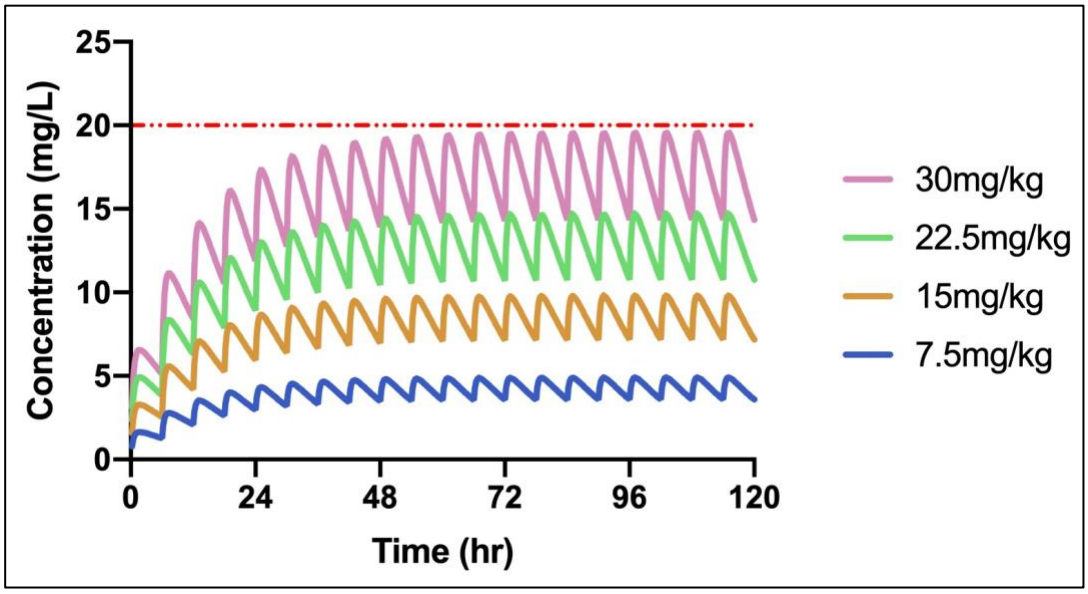

Figure 11: Computationally predicted neurotoxicity in horse model. *A neurotoxicological threshold is applied at 20 mg/L of the predicted simulation of central nervous system concentrations of metronidazole. The graph represents a predicted simulation of central nervous system concentrations of metronidazole with repeated dosing of an oral tablet given every 6 hours in an equine model.*

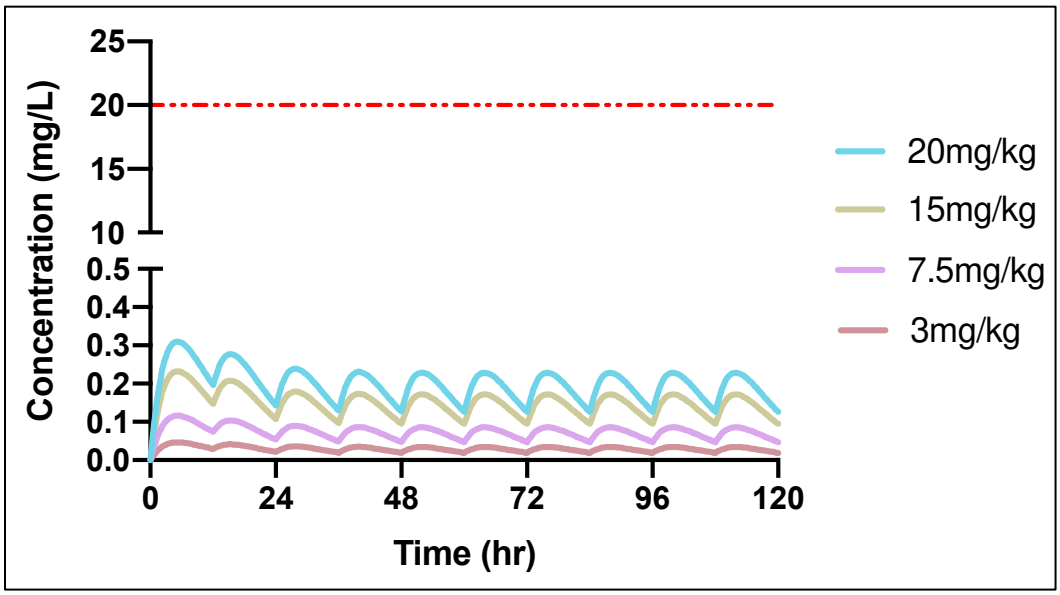

Figure 12: Computationally predicted neurotoxicity in mouse model*. A neurotoxicological threshold is applied at 20 mg/L of the predicted simulation of central nervous system concentrations of metronidazole. The graph represents a predicted simulation of central nervous system concentrations of metronidazole with repeated dosing of an oral tablet given every 12 hours in a murine model.*

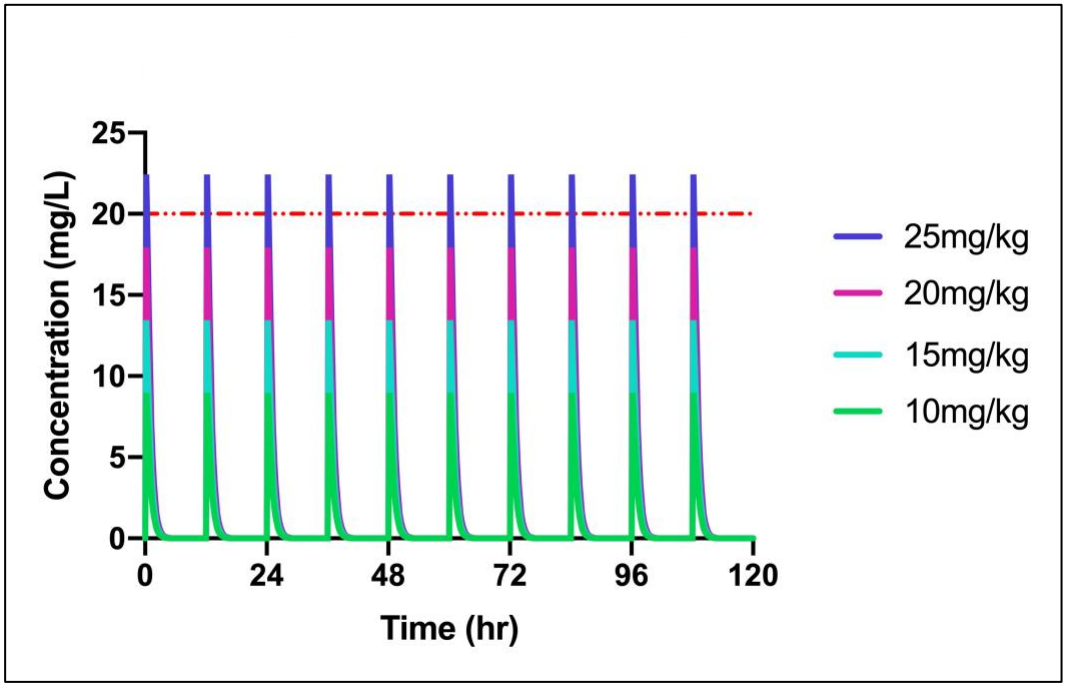

Figure 13: Computationally predicted neurotoxicity in rabbit model*. A neurotoxicological threshold is applied at 20 mg/L of the predicted simulation of central nervous system concentrations of metronidazole. The graph represents a predicted simulation of central nervous system concentrations of metronidazole with repeated dosing of an oral tablet given every 12 hours in a rabbit model.*

2.12 Doses of metronidazole did not cause glial death

To better understand the affected cell populations, a mixed glial culture was used to measure cell viability (Figure 14). MTT cell viability reagent was used to assess primary glial cells with the treatment of metronidazole. Glial cells were cultured in the absence and presence of 10 µg/ml, 20 µg/ml, 30 µg/ml, 40 µg/ml, 50 µg/ml, and 60 µg/ml concentrations of metronidazole for 72 hours. With increasing concentrations of metronidazole, our results demonstrate that optical density values remain about the same. Our results indicate that the cell populations did not experience cell death in a dose-dependent manner with treatments of metronidazole alone. Compared to the no treatment control, there is no change in treatment groups and no significance was seen. Significance was measured with a one-way ANOVA test ($p<0.05$).

We would expect that metronidazole treatments are not inherently toxic to the cell due to clinical cases of this neurotoxicity not showing long-term complications with the use of this drug (Kuriyama et al. 2011).

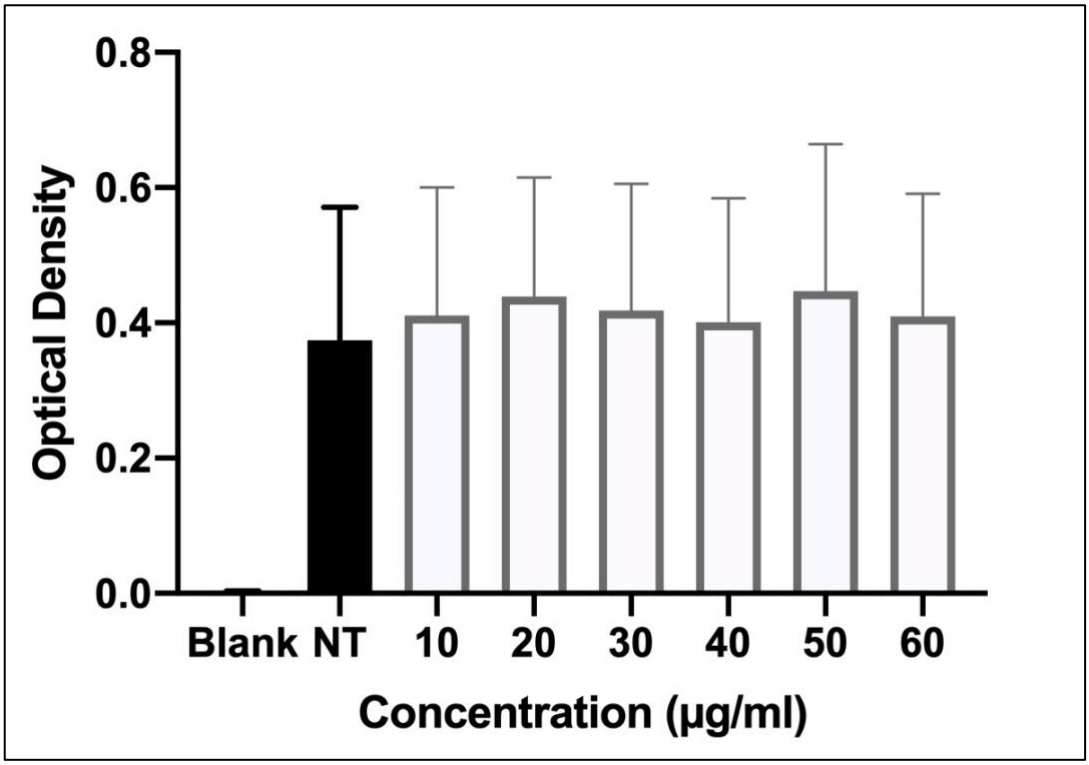

Figure 14: Cell viability assay demonstrates no cell death. *Using MTT cell viability reagent, primary murine mixed glia were treated with 10 µg/ml, 20 µg/ml, 30 µg/ml, 40 µg/ml, 50 µg/ml, and 60 µg/ml concentrations of metronidazole. Results were read on a VersaMax at 72 hr. Blank was used to correct for background fluorescence. NT = no treatment. Error bars represent standard deviation. No significance was seen between treatment groups using one-way ANOVA. Results are N = 2 technical replicates then ran in biological duplicates.*

2.13 Minimal changes to cholesterol levels

In order to establish the link between cholesterol in the brain and metronidazole, we utilized AMPLEX® red assay kit to measure total cholesterol levels of mixed glial cells. Cells were treated with metronidazole for 72 hours with 10 µg/ml, 20 µg/ml, 30 µg/ml, 40 µg/ml, 50 µg/ml, and 60 µg/ml concentrations. Overall, we see no change in cholesterol levels and no significance between any group. Significance was determined by a one-way ANOVA ($p<0.05$). Cholesterol was measured in ng/µg of loaded protein as previously defined (Nunes et al. 2013). Our results indicate that metronidazole alone is not enough to perturb cholesterol synthesis and leads us to our theory that it needs to be reduced to take an effect. The reduction of the nitro group on its imidazole ring is its mechanism of action in bacteria and fungi (Kelly 2001 and Sigeti 1983), however, this has yet to be established in mammalian systems.

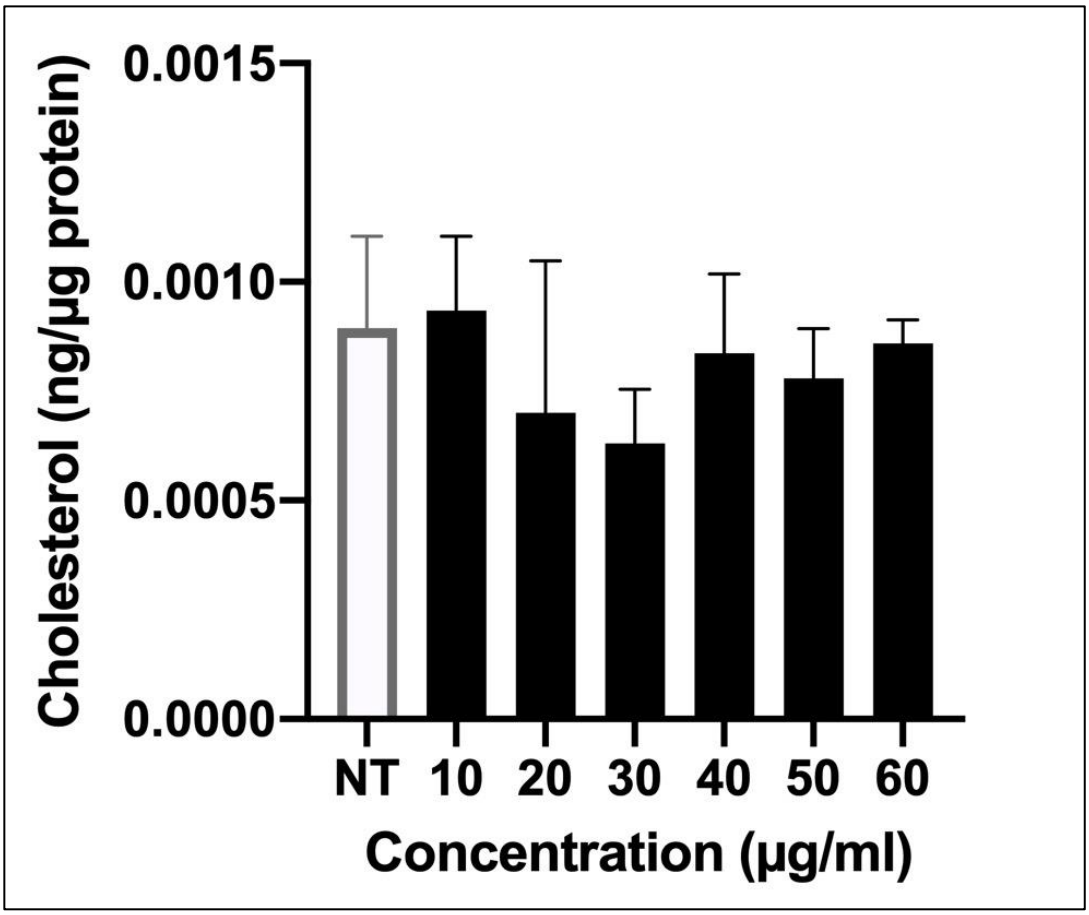

Figure 15: Total cholesterol levels demonstrate marginal changes with metronidazole treatment*. With the cholesterol AMPLEX® Red Assay kit, primary murine mixed glia were treated with 10 µg/ml, 20 µg/ml, 30 µg/ml, 40 µg/ml, 50 µg/ml, and 60 µg/ml concentrations of metronidazole, respectively. NT = no treatment. Error bars represent standard deviation. No significance was seen between treatment groups using one-way ANOVA. Results are N = 2 technical replicates then ran in biological duplicates.*

2.14 Liquid chromatography/Mass spectrometry

To establish a standard, the parent compound was identified per the materials and methods section. Further, we went on to establish the identification of the reduced metabolite (Figure 16). Utilizing sodium dithionite, a strong reducing agent, we initiated a reduction reaction with metronidazole in astrocyte media. Dingsdang et al. describe a proposed pathway of metronidazole reduction that results in a reactive metabolite (Dingsdang et al. 2018). Using this information, we found the molecular weight of the 5-amino compound and what we here describe as the reduced metabolite of metronidazole. It is unlikely we would be able to find the radical anion due to its short-lived lifespan. The 5-amino would be more stable and we were able to find it by its molecular weight of ~142 g/mol (see Figure 16). Due to its smaller size in comparison to the parent compound, it eluted from the column much quicker and can be seen at around the 1.5 minute mark on the chromatogram. The second peak that is seen there is metronidazole.

With our established parent and metabolite compounds, our future directions would be to take treated media from cells and utilize LC/MS to determine if metronidazole is being reduced *in vitro*.

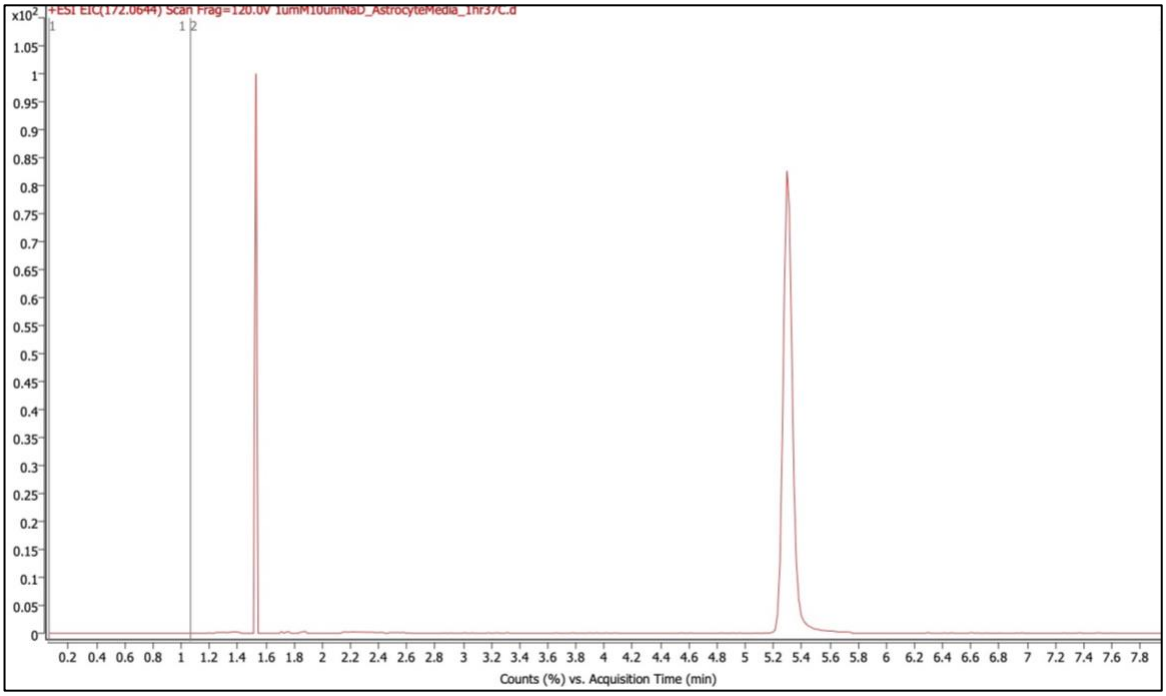

Figure 16: LC/MS identifies the reduced metabolite of metronidazole. *At timepoint 1.5 the reduced metabolite can be seen on this chromatogram. Through an extracted ion chromatogram (EIC), we are able to select for the metabolite via is molecular weight which is expected to be about 142 g/mol. The reduction environment consisted of 1 µm to 10 µm concentrations of metronidazole and sodium dithionite, respectively. This reduction occurred in astrocyte media, as described in materials and methods, and was read after 1 hour at 37º Celsius.*

2.15 LPS induced vulnerable model

With the treatment of LPS prior to antibiotic treatment, we create a vulnerable model of oxidative stress-induced neuroinflammation to reduce metronidazole in the glial cells. Cell viability and levels of cholesterol are reassessed in this model as previously described with additional treatments of 500 ng/ml of LPS in combination with metronidazole. In addition, RT-PCR was used to measure expression levels of products in the cholesterol biosynthesis pathway.

In looking at the cell viability assay in Figure 17, our results do not show cell death. We would similarly expect this due to the lack of long-term adverse effects from taking metronidazole, as previously stated.

For the cholesterol assay in Figure 18, our results demonstrate a decrease in total cholesterol for treatment groups, although not significant. We would expect with LPS treatment, that metronidazole would be reduced, and cholesterol metabolism would then be perturbed as the drug covalently binds to CYP51. However, we do not know if the perturbations in cholesterol metabolism are a result of changed expression levels of CYP51. Therefore, we implemented RT-PCR to investigate this.

In Figure 19, our results show that there are no changes to CYP51 expression levels after LPS and metronidazole combined treatments. We see no significance between treatment and control groups.

However, in Figure 20 we do see significance between the no treatment and metronidazole only groups. Our results demonstrate an upregulation of the expression of HMGCR, a rate-limiting step in the cholesterol biosynthesis pathway, and an upstream product in the pathway.

Additionally, in Figure 21, we see significance among all groups. Our results display the downregulation of APOE among treated groups. APOE is involved in trafficking cholesterol and is a downstream product of the biosynthesis pathway.

These results taken in combination with the metronidazole-only treatment groups give us a more in-depth look into cholesterol regulation within the cell. Future directions involve looking at cholesterol products immediately up and downstream to CYP51. This would give us a better idea of where the dysregulation is occurring.

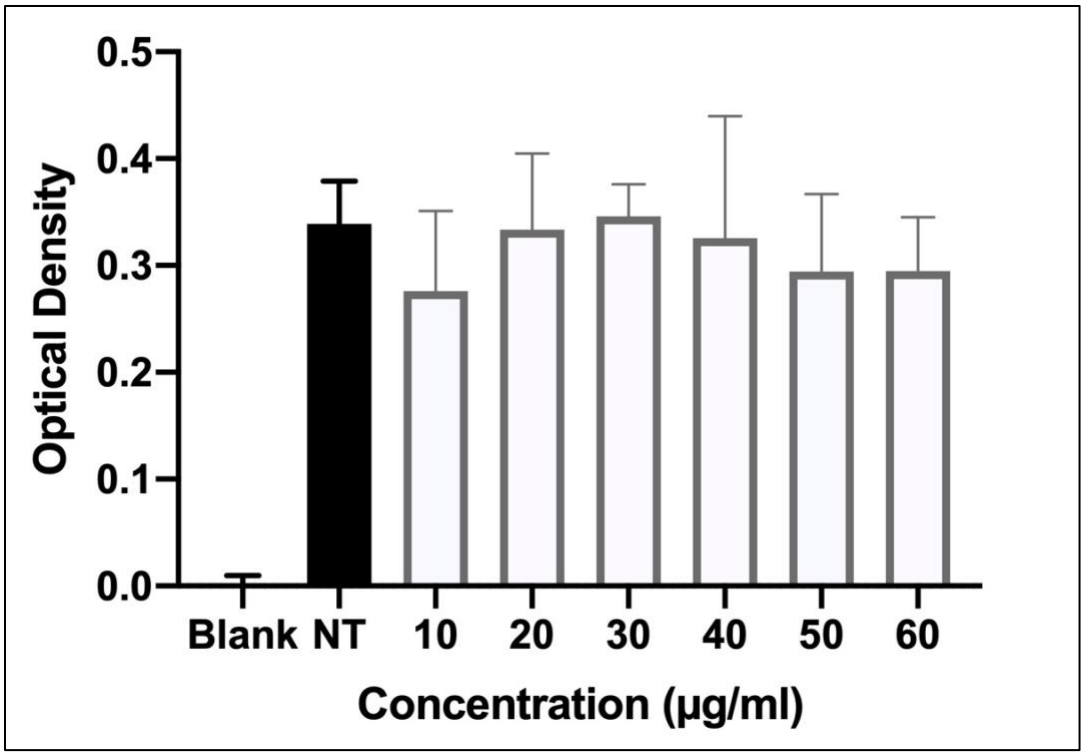

Figure 17: Cell viability demonstrates lack of cell death with combined LPS and metronidazole treatments*. Using MTT cell viability reagent, primary murine mixed glia were treated with 500 ng/ml concentration of LPS and then 10 µg/ml, 20 µg/ml, 30 µg/ml, 40 µg/ml, 50 µg/ml, and 60 µg/ml concentrations of metronidazole, respectively. Results were read on a VersaMax at 72hr. Blank was used to correct for background fluorescence. NT = no treatment. Error bars represent standard deviation. No significance was seen between treatment groups using one-way ANOVA. Results are N = 2 technical replicates then ran in biological duplicates.*

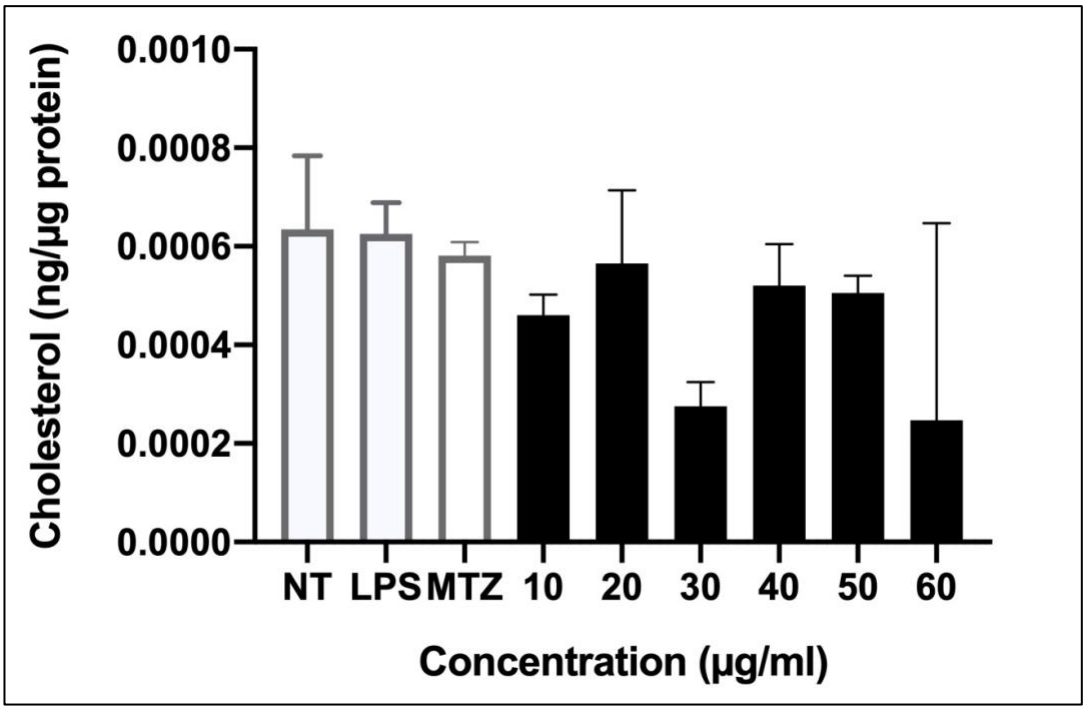

Figure 18: Total cholesteroal levels demonstrate marginal changes with combined LPS and metronidazole treatments. *With the cholesterol AMPLEX® Red Assay kit, primary murine mixed glia were treated with 500 ng/ml of LPS and then 10 µg/ml, 20 µg/ml, 30 µg/ml, 40 µg/ml, 50 µg/ml, and 60 µg/ml concentrations of metronidazole, respectively. NT = no treatment, LPS = 500 ng/ml concentration of LPS only, and MTZ = 60 µg/ml concentration of metronidazole only. Results were read on a VersaMax. Error bars represent standard deviation of technical replicates. No significance was seen between treatment groups using one-way ANOVA. Results are N = 2 technical replicates.*

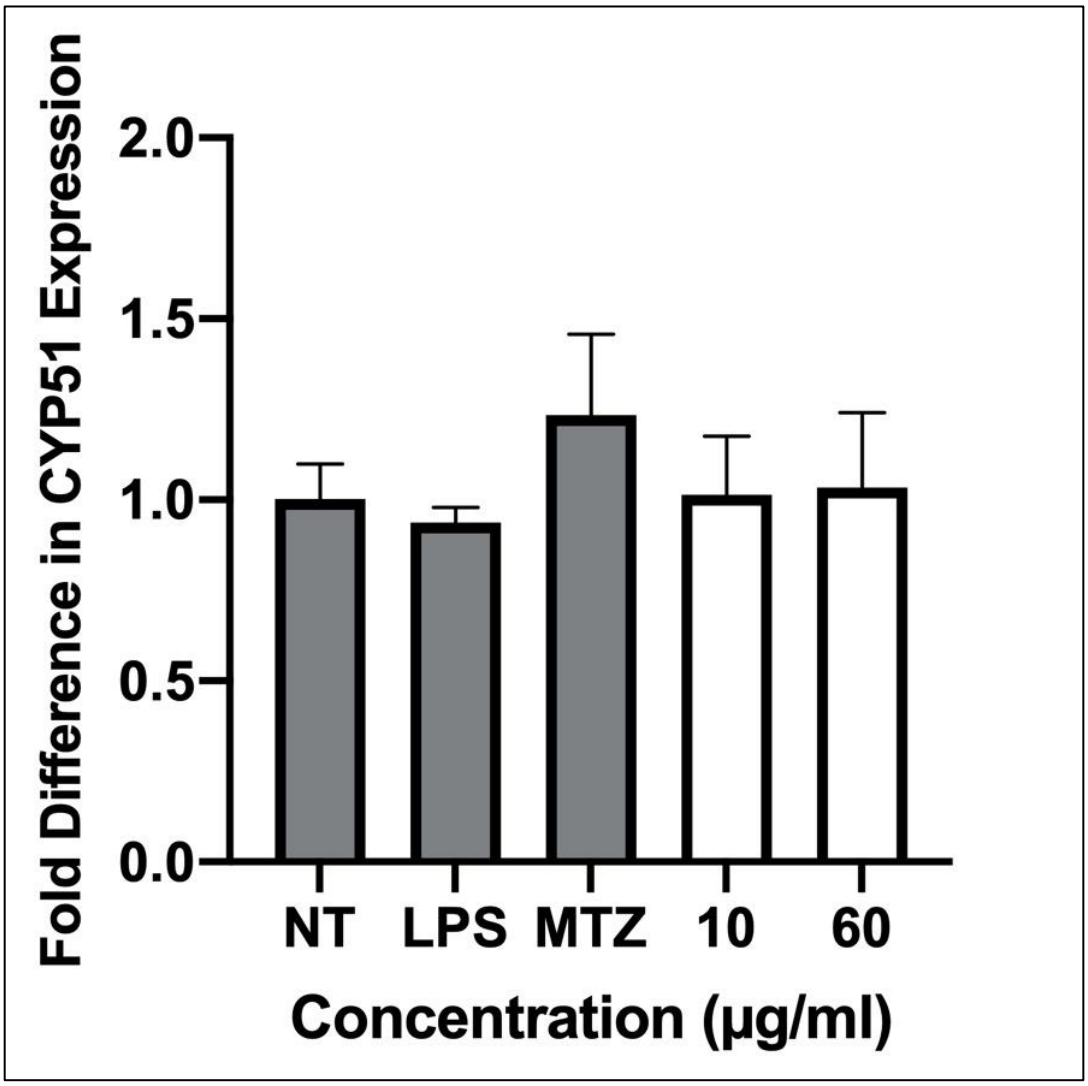

Figure 19: No change in CYP51 expression levels. *iQ™SYBR® Green Supermix was used to assess primary murine mixed glia for RNA expression levels. Cells were treated with 500 ng/ml of LPS and then 10 µg/ml and 60 µg/ml concentrations of metronidazole, respectively. NT = no treatment, LPS = 500 ng/ml concentration of LPS only, and MTZ = 10 µg/ml concentration of metronidazole only. Results were read on a VersaMax. Error bars represent standard deviation of technical replicates. No significance was seen between treatment groups using one-way ANOVA. Results are N = 2 technical replicates.*

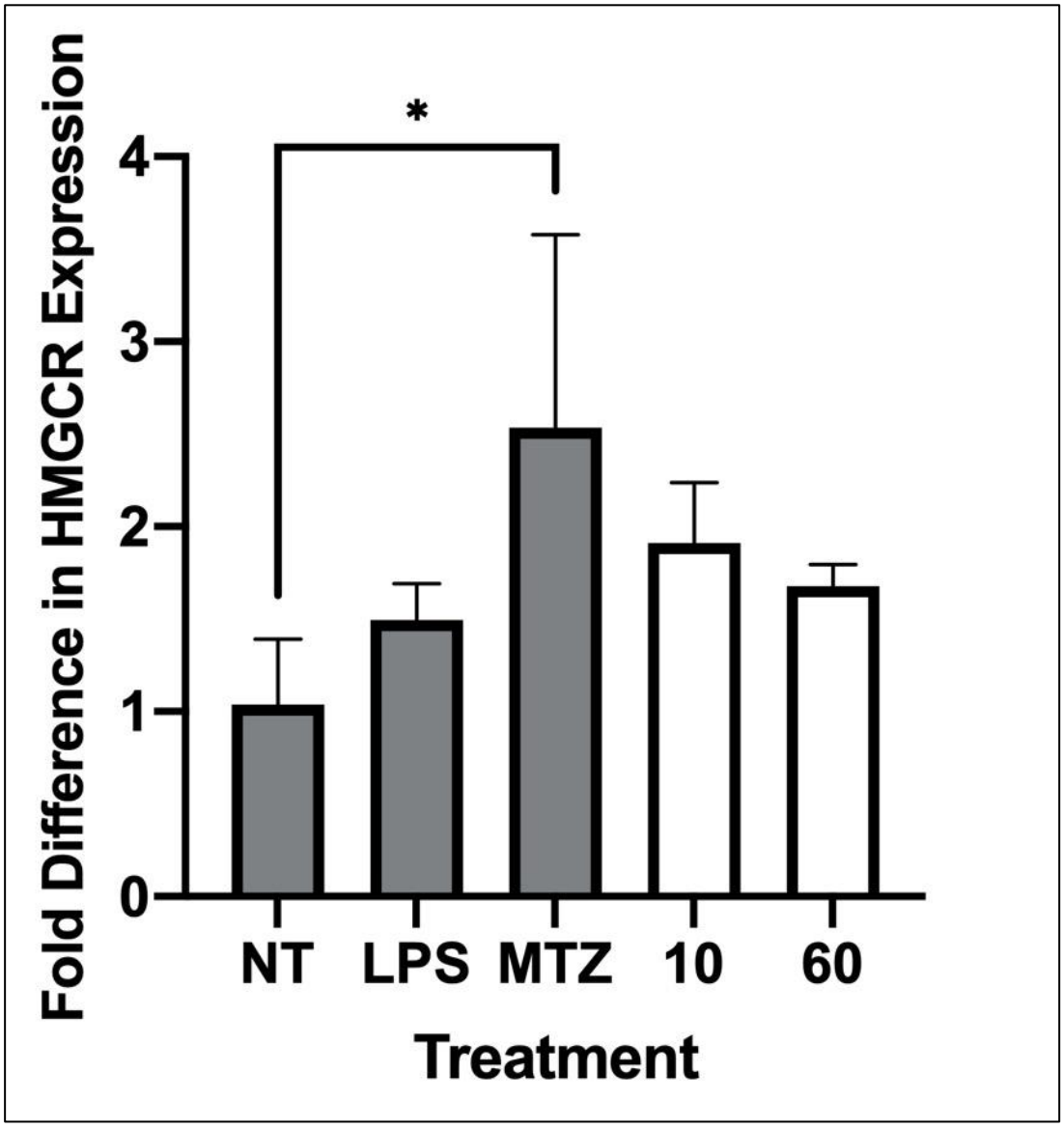

Figure 20: An increased expression of HMGCR in metronidazole treated cells. . *iQ™SYBR® Green Supermix was used to assess primary murine mixed glia for RNA expression levels. Cells were treated with 500 ng/ml of LPS and then 10 µg/ml and 60 µg/ml concentrations of metronidazole, respectively. NT = no treatment, LPS = 500 ng/ml concentration of LPS only, and MTZ = 10 µg/ml concentration of metronidazole only. Error bars represent standard deviation of technical replicates. Significance was seen between treatment NT and MTZ groups using one-way ANOVA. Results are N = 2 technical replicates.*

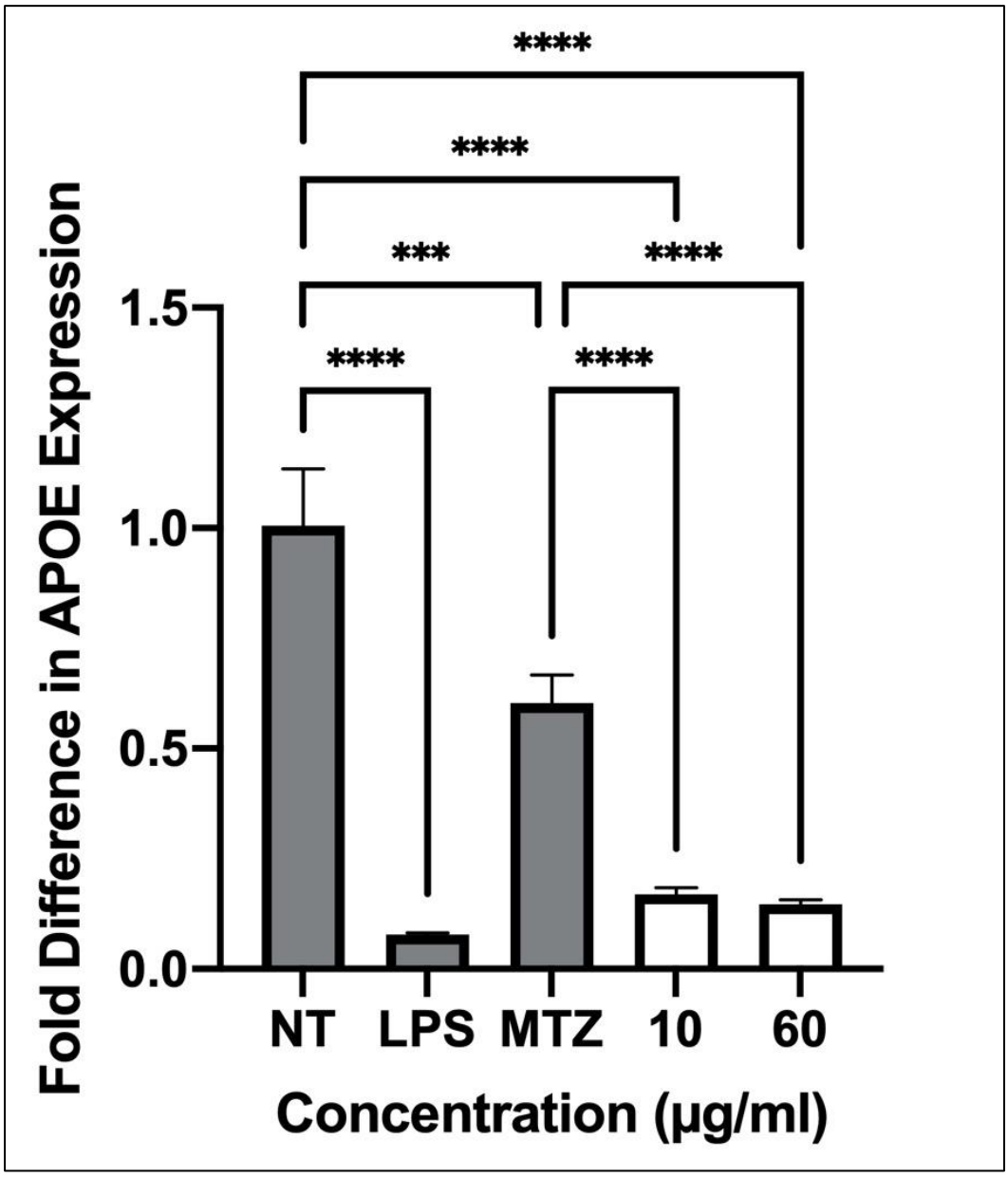

Figure 21: Decrease in expression of APOE in LPS and metronidazole treated cells. . *iQ™SYBR® Green Supermix was used to assess primary murine mixed glia for RNA expression levels. Cells were treated with 500 ng/ml of LPS and then 10 µg/ml and 60 µg/ml concentrations of metronidazole, respectively. NT = no treatment, LPS = 500 ng/ml concentration of LPS only, and MTZ = 10 µg/ml concentration of metronidazole only. Error bars represent standard deviation of technical replicates. Significance was seen between all treatment groups using one-way ANOVA. Results are N = 2 technical replicates.*

2.16 Established PBPK models update with novel information

Finally, as computational modeling is an evolving platform, we can now update the simulation with new experimental data in order to provide a more accurate model of neurotoxicity. For Figures 24-27, an additional threshold has been applied at 10.1mg/L concentration. This line represents the inhibitory concentration (IC) at which 50% of molecules of CYP51 would be inhibited by metronidazole. The results reveal a much lower theoretical neurotoxicological threshold with this newly applied line and more accurately describes low-dose neurotoxicity seen clinically (Kuriyama et al. 2011).

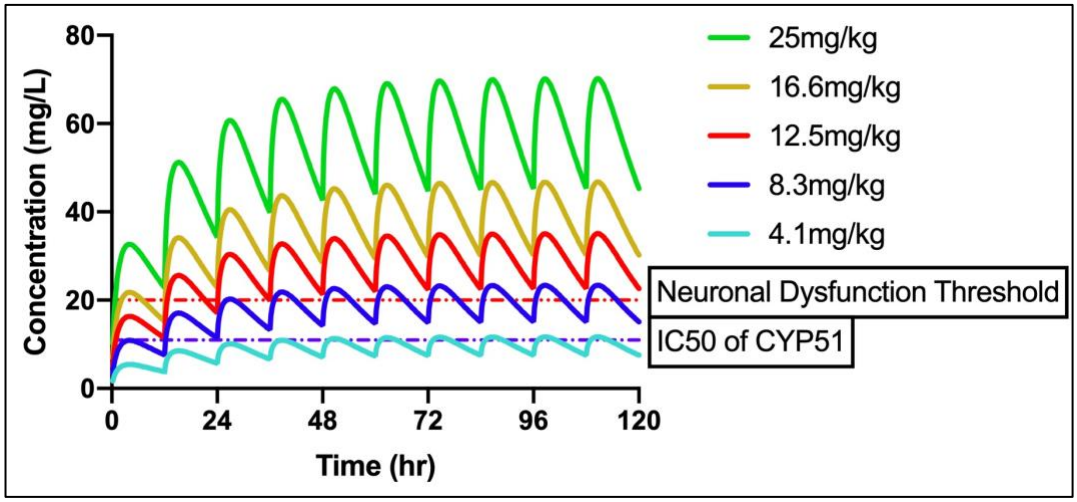

Figure 22: Updated predicted neurotoxicological model with CYP51 for human model. *A neurotoxicological threshold is applied at 20mg/L of the predicted simulation of central nervous system concentrations of metronidazole with repeated dosing regimen given every 12 hours in a human model, over a five-day time scheme. An additional neurotoxic line is drawn at 10.1mg/L to represent the IC50 of CYP51 with metronidazole.*

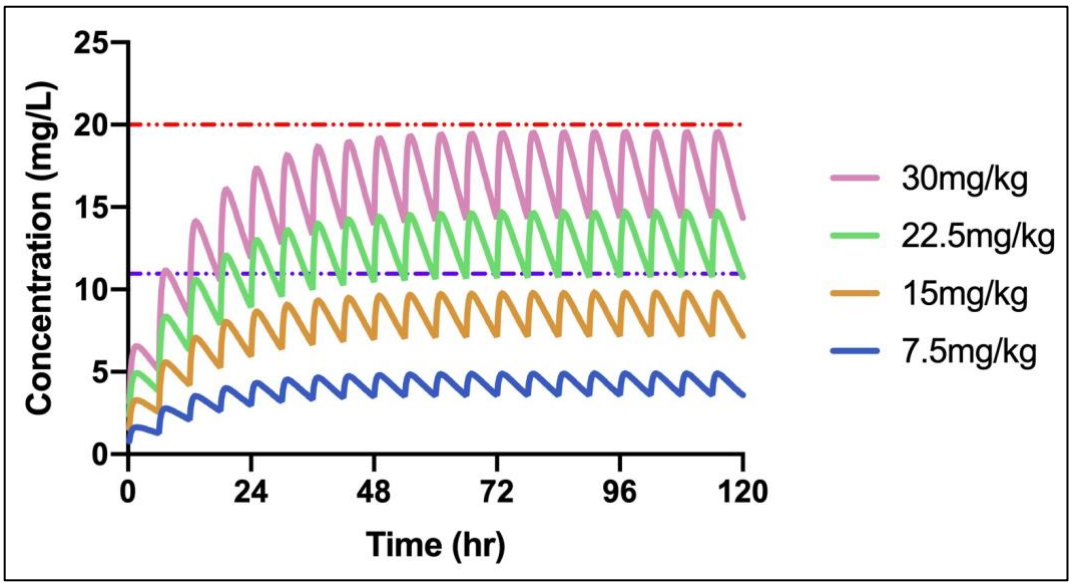

Figure 23: Updated predicted neurotoxicological model with CYP51 for horse model. *A neurotoxicological threshold is applied at 20mg/L of the predicted simulation of central nervous system concentrations of metronidazole with repeated dosing regimen given every 6 hours in a horse model, over a five-day time scheme. An additional neurotoxic line is drawn at 10.1mg/L to represent the IC50 of CYP51 with metronidazole.*

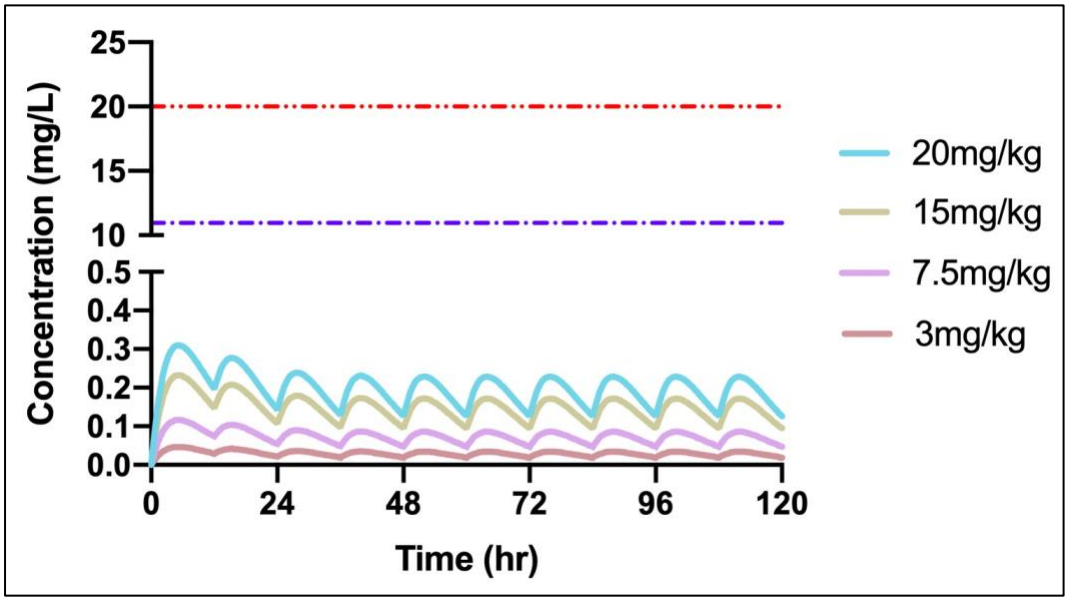

Figure 24: Updated predicted neurotoxicological model with CYP51 for mouse model. *A neurotoxicological threshold is applied at 20mg/L of the predicted simulation of central nervous system concentrations of metronidazole with repeated dosing regimen given every 6 hours in a horse model, over a five-day time scheme. An additional neurotoxic line is drawn at 10.1mg/L to represent the IC50 of CYP51 with metronidazole.*

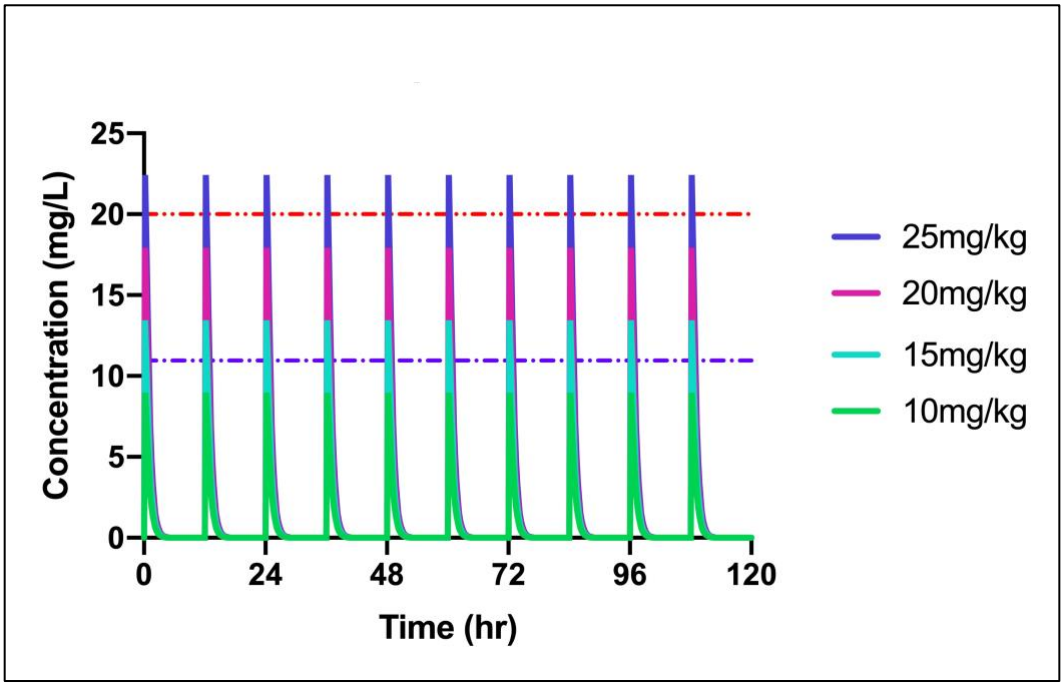

Figure 25: Updated predicted neurotoxicological model with CYP51 for rabbit model. *A neurotoxicological threshold is applied at 20mg/L of the predicted simulation of central nervous system concentrations of metronidazole with repeated dosing regimen given every 12 hours in a rabbit model, over a five-day time scheme. An additional neurotoxic line is drawn at 10.1mg/L to represent the IC50 of CYP51 with metronidazole.*

Discussion

Computational-based simulation modeling is becoming a more prevalent predictive tool in scientific research. The need for more efficient and accurate tools that reduce the use of live animal models may be more beneficial over long-term use in the current climate of animal welfare and ethics.

The current study is novel in that it evaluates both human and animal model dosing schemes, to improve quantification of dosing regimens of metronidazole. These models provide correlational toxicological data that better quantifies oral dosing regimens that may result in adverse neurological outcomes and allows for mechanistic inferences. Although, it is important to note that our results may be variable due to our use of mixed female and male rodent neuron and glial cultures and the implicated hormonal differences with steroids. Computational modeling is unique such that it can be supplemented as new information rises and subsequently results in a better predictive model. This simulation model has allowed us to derive important clinical outcomes without the use of live animal models. In addition, these models demonstrate visible physiological differences between species and have allowed us to see that, compared to a human model, a murine model would likely poorly mimic the neurotoxicological outcomes of metronidazole toxicity due to their high resistance to toxicity. This is corroborated with their toxicity profile data because it shows the LD50 of oral administrations of metronidazole in rats to be 3 g/ml (Cavalleri et al. 1977). Additionally, none of the animal models accurately represent the high brain partition coefficient values reflected in the human model. More investigative research can be done in addition to more simulation work with different animal models, provided experimental data exists.

Our cholesterol analysis demonstrates that the cholesterol biosynthetic pathway is modulated by both LPS and metronidazole. However, further interpretation of the results demonstrates that in addition to modulating cholesterol synthesis, metronidazole exhibits a disruption of it. Treatment of glial cells with LPS and then metronidazole decreased mRNA expression of apolipoprotein E (APOE) and increases the mRNA expression of 3-hydroxy-3-methyl-glutaryl-coenzyme A reductase (HMGCR) (Figures 20 and 21). However, when looking at broader cholesterol, metronidazole shows lowered levels of synthesis (Figure 18). LPS indiscriminately perturbs and damages other proteins within the cell likely due to reactive oxygen species production (Zhao et al. 2019). However, this nonspecific damage allows the cell to upregulate cholesterol biosynthesis pathways in response to this and repair itself (Feingold et al. 1993). Contrarily, because metronidazole has a high specificity to a protein in the cholesterol biosynthetic pathway, it covalently binds to CYP51 and, because of this, the cell is unable to compensate resulting in lowered cholesterol levels. Our unchanged CYP51 expression levels elude to metronidazole forming a protein adduct that temporarily inhibits the enzyme structure and/or function. Furthermore, the conserved nature of CYP51 gives additional explanation to this neurotoxic clinical outcome happening in multiple species. And due to metronidazole's inherent bacteriostatic nature, this may also prove to explain the "reversible" findings when the drug use is discontinued (Kuriyama et al. 2011).

LPS treated mice via i.c.v. injections induced neuroinflammation and subsequent cognitive impairment in C57BL/6J mice in a study done by Zhao et al. The authors used high concentrations of LPS, and this resulted in the mice exhibiting similar behaviors to later staged neurodegeneration that is seen in humans (Zhao et al. 2019). Our work here

was *in vitro* and with significantly lower concentrations of LPS. With these lower concentrations, we have demonstrated dysregulation of cholesterol metabolism, therefore, our results elude to further research that can be done to correlate vulnerable populations to metronidazole neurotoxicity and early neuroinflammatory neurodegeneration.

The kinetics of the reduction of metronidazole is something else to consider in our proposed theory. Conflicting reports exist on whether metronidazole causes DNA damage or DNA inhibition (LaRusso et al. 1978 and Knight et al. 1978). Being an antibiotic, metronidazole is largely activated in anaerobic low reductive potential environments. It is important to note, in order for metronidazole to actively induce injury, it must be reduced. Here we describe a vulnerable model created from oxidative stress to provide an environment conducive to metronidazole's reduction and have future experiments planned to corroborate this via LC/MS. A threshold of oxidative stress must be met in order to demonstrate this and future studies can be done to investigate this minimum threshold. Other antibiotics with similar structures as metronidazole have been cited as neurotoxic, particularly ronidazole in cats (Rosado 2007). Due to ronidazole's structural similarity and nitro group, we would expect similar results as seen with metronidazole treatment. While metronidazole has been a drug of choice for decades, the mechanism of its unintended neurotoxicity is not known and is poorly researched. The data here gives rise to interest in investigating this phenomenon as it could prove useful in the identification of vulnerable human and animal species. It is also important to note that here we are describing an explanation to low dose neurotoxic outcomes. High doses of any chemical will result in a toxic effect but in clinical cases such as described in Kuriyama

et al. human patients on as little as a 250 mg oral tablet taken for one day, also experienced adverse outcomes from this drug (Kuriyama et al. 2011). As the simulated models predicted, doses as low as 250 mg coincide with the predicted IC50 of CYP51.

Interestingly, an article from 1987 found metronidazole and other nitroheterocyclic drugs to be reduced by catecholamine neurotransmitters such as norepinephrine, dopamine, and related molecules (Ramakrishna 1987). Both semiquinone radicals and nitro anion radicals were seen through electron spin resonance spectroscopy in the stated article. This could provide a further link to the reduction of metronidazole in mammalian metabolic brain processes despite the mammalian environment not being as ideal as a bacterial environment.

Low doses of metronidazole can be potentially linked to adverse neurological effects in humans, unlike other animals. Future studies should be done *in vivo* with multiple species to cognize low dose neurologic effects of metronidazole. Further, the mechanism of this impaired cholesterol metabolism could prove important as a link to diagnosing early neurodegenerative processes.

Chapter 3

Discussion and conclusion

Neurotoxicity has a broad assortment of effects on many species that range from molecular to clinical outcomes. Notably, the key component to the elicitation of these adverse effects is the biotransformation of these drugs. Metronidazole (MTZ) neurotoxicity occurs through a chemical reduction of the nitro group present on its imidazole ring. Subsequent to this reduction, a reactive metabolite forms, and metronidazole then acts as an alternate electron acceptor potentially disrupting normal cellular metabolic processes. As an anti-fungal, MTZ has a high binding affinity for CYP51 and is known to be a potent inhibitor to this highly conserved cholesterol-related protein. While MTZ neurotoxicity can manifest pathologically through several different mechanisms, we mainly focus on a novel connection that disrupted glial cholesterol metabolism is the culprit. Here we have described a cholesterol-mediated mechanism that accounts for common adverse incidences in multiple species with the use of this drug such as central and peripheral neurotoxicities as well as low-dose adverse outcomes (Sagvekar et al. 2019).

In our simulation work, the PBPK models of the human, horse, rabbit, and mouse demonstrated varying predicted neurotoxicological outcomes with similar therapeutic doses of MTZ. Nevertheless, differences in the tissue availability of the key enzymes involved in the biotransformation of MTZ could partly explicate the vastly different predicted elimination constants shown between them. In humans, MTZ is largely metabolized through hydroxylation, a biotransformation process that involves enzymes

called hydroxylases catalyzing a reaction to add a hydroxyl group to a substrate in order to help it be more readily excreted through the kidneys. However, in other species, such as mice, glucuronidation is the primary reaction to excrete MTZ and details glucuronosyltransferases catalyzing the addition of glucuronide to a substrate instead. In the brain tissue of humans, UDP-glucuronosyltransferase (UGT) is widely expressed but at relatively low levels compared to the liver (Zhang et al. 2016), and the main hydroxylation metabolizer of MTZ, CYP2A6, is expressed at very low levels or not at all (Li et al. 2017). This could explain its poor preferential detoxification in the human brain and why neurotoxicity is more likely to occur. Consequently, the drug's ability to penetrate the blood-brain barrier coupled with poor tissue enzyme availability and high predicted brain-to-blood partition coefficient results in slow metabolization and ultimately increases the chance of other reactions occurring, e.g., reduction to toxic metabolite.

As previously mentioned, a well-researched hallmark of early neurodegeneration is an increase in oxidative stress among various other mechanisms. However, early neurodegeneration from oxidative stress is a mechanism that is largely undetected until substantial accumulation occurs and, by then, it is considerably in its later stages and typically resistant to therapeutic interventions. Discovering a way to detect early neurodegeneration would allow for the implementation of early pharmacological interventions to rescue or slow the onset of disease in humans and animals. With MTZ's ability to cross the blood-brain barrier and unique reductive characteristics, reduction via early neurodegenerative-related oxidative stress is a plausible theory. Furthermore, its reversible nature, current drug safety profile, and non-invasive means make the benefits far outweigh the risks for its potential use as a novel diagnostic tool. Although we largely

discuss neurodegeneration, oxidative stress is not exclusive to this pathology and could be an indication of other neurologic outcomes such as aging, neurotoxicity, stress, etc. This makes it imperative that more research is done to investigate this reaction and further identify the specificity at which it occurs. To demonstrate, an article by Ramakrishna Rao found that nitroimidazoles, similar to and including MTZ, were reduced by catecholamine neurotransmitters in alkaline solutions (Ramakrishna Rao et al. 1987). This is interesting because catecholaminergic cell groups of the central nervous system have been linked to Parkinson's disease (PD) and are known to generate increased levels of oxidative stress with disease and aging. In addition, catecholamine neurotransmitters are present in the central and peripheral nervous systems, both areas in which MTZ has clinically demonstrated neurotoxic effects. Furthermore, it has also been found that catecholamines exacerbate amyloid β-peptide neurotoxicity which plays a key role in Alzheimer's disease pathology (Fu et al. 1998), and *in vivo* increases to dopamine can oligomerize with α-synuclein and mimic synuclein pathologies seen in PD (Mor et al. 2017, Post et al. 2018, and Pieri et al. 2016). In the article by Fu et al., after neurons were exposed to amyloid-beta (Aβ) deposits, cytoplasmic and mitochondrial oxidative stress and lipid peroxidation were increased. Introducing catecholamines worsened this outcome and higher concentrations even induced mitochondrial dysfunction. Moreover, dopamine-derived α-synuclein oligomers were found to self-propagate as well as cross-propagate Aβ aggregates (Planchard et al. 2014) further demonstrating dopamine's ability to exacerbate effects. Catecholamines' can be oxidized by MTZ in addition to their molecular role in generating and exacerbating oxidative stress mechanisms could prove to be the molecular mechanistic relation to unintended clinical MTZ-induced neurotoxicity.

Additionally, increased levels of 3-Nitrotyrosine, an indicator of oxidative stress, and nitrated α-synuclein were found in the pars intermediate of horses with pituitary pars intermedia dysfunction (PPID), also known as Cushing's disease (Ambrojo et al. 2018 and McFarlane 2011). The pathology of PPID involves poor neuronal dopamine production leading to low levels in the pars intermedia of the pituitary gland likely resultant from free radical formation and oxidative stress. Due to overlapping similarities between PD and PPID equines may demonstrate the potential to serve as a model for human PD (McFarlane 2007) and our computational modeling work provides additional support for human and equine neurodegenerative translational research. Notably, Bandookwala and Sengupta suggest that 3-Nitrotyrosine may be a potential biomarker for neurodegeneration. Through a literature review, 3-Nitrotyrosine was found to be elevated in association with Huntington's, Lou Gehrig's, Prion, Alzheimer's, and Parkinson's Diseases (Bandookwala et al. 2020). 3-Nitrotyrosine is produced by an increase in nitric oxide production by glia leading to resultant superoxide interactions producing peroxynitrite which then nitrates protein tyrosine residues (Moreno et al. 2009). As a precursor to dopamine, tyrosine nitration can be an illuminating indication of increased oxidative stress in catecholamines.

With these prominent links to oxidative stress, it is unsurprising that the most recently proposed mechanism of neurotoxicity is an increase of oxidative stress factors such as reactive oxygen species, nitric oxide, and TNF-α leading to inflammation and an apoptotic cascade resulting in MTZ-mediated neurodegeneration (Chaturvedi et al. 2020). However, the high-dose treatments done in this work do not fully account for low-dose neurotoxicity described clinically in the literature. Our simulation work demonstrates

that *in vivo* murine models would be unsuitable to investigate this mechanism due to the extremely high doses necessary and poor translation to human and other mammals described with MTZ-neurotoxicity. Here we suggest that rabbits and horses more closely resemble human outcomes but, nevertheless, have a working model ready to be updated to simulate new animals as future data permits.

While oxidative stress is a favorable component of neurodegeneration, and notable to this current theory, it has been mentioned that cholesterol plays important pathological roles in the central and peripheral nervous systems as well. Most relevant to us is lanosterol 14-α-demethylase (CYP51) a precursor to cholesterol in its biosynthetic pathway. Neurodegenerative disease pathology includes an increased accumulation of cholesterol in brain tissues with the decreased expression of cholesterol-24 hydroxylase (CYP46A1) an enzyme involved in its elimination, similar to APOE. There is some evidence of azoles being potent binders to CYP46A1. However, it seems as though MTZ's structural uniqueness with the nitro group is not included in this group. Due to the idea of drug treatments targeting cholesterol synthesis to decrease cholesterol levels (Nunes et al. 2013), it may seem that MTZ could also have use as a drug therapeutic for patients with AD or other cholesterol accumulating neurodegenerative disease. For those without intracellular cholesterol accumulation, MTZ may disrupt myelin membranes leading to adverse clinical effects. This may be plausible due to 70% of cholesterol in the CNS residing in the myelin sheaths of neurons. If MTZ is disrupting cholesterol metabolism, this would be the largest source available to do so. Although contrary to metronidazole, miconazole, a related drug but without a nitro group, is cited as being protective and promotes the remyelination of axons largely through inhibition of

CYP51(Najm2015, Wang 2019). Moreover, all of this in addition to our work contributes to substantiating evidence in favor of this proposed cholesterol theory and may even rationalize conserved mechanistic neurotoxicity mediated by MTZ and catecholamines.

A final key piece to this obscure puzzle is the nitro group on MTZ's imidazole ring. In bacteria, nitroreductases catalyze the reduction of this constituent resulting in a short-lived and unstable radical metabolite that then binds and inhibits metabolic processes. Mammals lack this major reductive catalyzing enzyme as well as low reductive and anaerobic environments commonly found in bacteria that are more conducive to activating MTZ. In low reductive potential environments, the kinetics of MTZ are higher which can lead to increased damage to metabolic processes as well as increased protein and DNA adducts, making it an effective antibiotic. However, due to the aerobic environment of the mammalian brain, it is reasonable that MTZ would not be able to cause DNA fragmentation in this manner. The two authors who saw DNA fragmentation with MTZ treatment likely re-created an optimal environment that increased the kinetics of this reaction (Knight et al. 1987 and Tocher et al. 1992). For the experiments, they utilized electrolytic reduction at a voltage constant of -900mV. It is cited that MTZ needs only a reductive potential of -415mV and/or -450mV for the nitro group to be reduced (Kaakoush et al. 2009 and Edwards 1980) making -900mV undue and would explain the exacerbated effects seen such as DNA fragmentation. In normal mammalian tissues, the environment is unlikely to present in an optimal state such as that. Most mammalian cells have a normal resting potential of -50mV which is largely why, in most circumstances, taking metronidazole is safe and shows its high specificity for bacteria. Be that as it may, there are always exceptions when it comes to the complexities of biological systems and, other

than our proposed mechanism, the microbiome may also be one to play a role. In recent literature, more evidence is coming out in favor of the gut-brain axis theory of which brain function can be modulated by gut microbiota and dietary consumption. In a study looking at diet-induced obesity mice, with and without antibiotic treatment, to better understand the gut microbiome and brain insulin resistance, one author found that treatment with MTZ decreased inflammation and improved insulin signaling in the brain. This additionally reduced signs of anxiety and depression (Soto et al. 2018). Moreover, Flowers et al. comprised a review detailing the potential effects gut microbiota can have on xenobiotic pharmacokinetics and bioavailability. While the reduction of MTZ is not confirmed, the authors list other drugs with known reductive biotransformation by gut microbiota. Furthermore, the authors note that MTZ has known interactions with *Clostridium perfringens*, which causes a decrease in MTZ's action. This could lead to the notion that vulnerable populations to this neurotoxicity could all share specific microbial profiles that demonstrate increased sensitivity to this drug or a potential role for microbial modulation of MTZ. Interestingly, the authors cite levodopa as being metabolized by the gut microbiota into dopamine. This resulted in decreased systemic levels of dopamine and, in turn, required higher doses of levodopa (Flowers et al. 2020). Although this is a promising hypothesis we would need to perform more research to fully understand the role of the microbiome on MTZ neurotoxicity.

All things considered, whether through the microbiome, catecholamines, and/or early generated oxidative stress MTZ's unintended reduction to act as an alternate electron acceptor is, here, believed to covalently bind to CYP51 and disrupt glial cell cholesterol metabolism resulting in poor axon myelination and subsequent adverse

clinical outcomes. The first step to this theory, establishing a connection between metronidazole and glial cholesterol metabolism, has been done and leads the way for further work to be done to elucidate the specific circumstances in which this occurs in the mammalian system.

In conclusion, further research should be done to substantiate this process *in vivo*, preferably with rabbit and equine models coupled with *in vitro* and clinical human work. With a better understanding of this cholesterol-mediated neurotoxicity, we can better predict vulnerable populations of humans and animals to MTZ in high and low dosing regimens. Furthermore, metronidazole may have connections to oxidative stress-related neurodegenerative processes while potentiating its own cholesterol-mediated neurotoxicity and therefore prove worthwhile to elucidate for the better understanding of other neurotoxicities.

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