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### Mitoxantrone Represses Markers of Microglial Activation and Inflammation

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# SENIOR THESIS APPROVAL

This Honors thesis entitled

## **Mitoxantrone Represses Markers of Microglial Activation and Inflammation**

written by

**Cameron A. Tull**

and submitted in partial fulfillment of the  
requirements for completion of the  
Carl Goodson Honors Program  
meets the criteria for acceptance  
and has been approved by the undersigned readers.

thesis director

second reader

third reader

honors program director

April 19, 2006

# **Mitoxantrone Represses Markers of Microglial Activation and Inflammation**

Cameron A. Tull

## **Abstract**

Multiple sclerosis (MS) is a neurodegenerative disease characterized by an autoimmune attack against myelin sheaths in the central nervous system (CNS). Resulting debilitations vary from sensory, motor, and coordination abnormalities to visual difficulties as well as bowel, bladder, sexual, and cognitive dysfunction (Fox, 2006). Mitoxantrone (Novantrone) is an FDA-approved drug used to treat the secondary-progressive form of MS due to its demonstrated immunosuppressive properties. While the mechanism of action of mitoxantrone is not yet well understood, and is limited in its use due to cardiotoxicity, the aim of this study was to determine the effect of mitoxantrone on microglial and astrocyte activation as a measure of the inflammatory response. Enzyme-linked immunosorbent assays (ELISA) showed that lipopolysaccharide (LPS) stimulates markers of activation including nitric oxide (NO), interleukin-1-beta (IL-1b), interleukin-6 (IL-6), and monocyte chemoattractant protein-1 (MCP-1) in N9 cultured microglia, and moreover, that mitoxantrone inhibits these effects in a dose-dependent manner.

## **Introduction**

### *MS*

The purpose of our research was to determine the specific effects of mitoxantrone on LPS-induced activated microglial cells to elucidate the pathways through which it alters the inflammatory response characteristic of secondary progressive multiple sclerosis.

Up to 2 million people are affected by multiple sclerosis world-wide, and approximately 300,000 to 350,000 individuals suffer from MS in the United States alone (Anderson et al., 1992).

Although women are twice as likely as men to develop MS (Fox, 2006), the disease is often more severe in men (Voskuhl and Palaszynski, 2001). MS may be diagnosed in people between the ages of twenty and sixty, but it is most commonly diagnosed between the ages twenty-five to forty years of age (Simone et al., 2000).

Multiple sclerosis is a chronic inflammatory disease of the central nervous system and is characterized as an autoimmune reaction against the myelin sheaths that surround the nerves. In MS the primary pathological target is myelin and it is this demyelination, oligodendrocyte loss, and axonal degeneration that lead to the subsequent loss of neurological function (Trapp, 1999). Patients affected by MS typically are found to have multiple lesions in cerebral white matter, and these areas of inflammation have been seen both in autopsy specimens as well as with the use of magnetic resonance imaging (MRI) (Fox, 2006). Lesions found in the brainstem, corpus callosum, juxtacortical area, or adjacent to the body of the lateral ventricles, are most specific for MS (Fox, 2006). Often these lesions are oriented perpendicularly to the lateral ventricle and are round or ovoid (Fox, 2006). There is indication of breakdown of the blood-brain barrier resulting from the development of MS lesions. This results in the invasion of hematogenous monocytes and T cells into the brain parenchyma (Trapp, 1999). The immune system is implicated in active tissue destruction by the association of inflammatory cells with new MS lesions (Trapp, 1999).

The most common form of multiple sclerosis usually strikes young adults and is termed classical MS. Classical MS is usually marked by two distinct phases: relapsing-remitting and secondary progressive. Initially, MS presents in relapsing-remitting form, a phase which can last 8-20 years before succeeding onto the secondary progressive stage, which is marked by continuously progressive disabilities of the nervous system (Trapp, June 1999). With the relapsing-remitting form of MS, periods of sporadic neurological deficits are intermittent with

variable periods of remission. There is no way to predict the onset of the secondary progressive phase, and the mechanisms by which relapsing-remitting converts into secondary progressive MS are not understood (Trapp, June 1999). Due to the fact that the vast majority of neurological disability in patients suffering from MS is accounted for in the secondary progressive phase, it is this phase that multiple sclerosis researchers are trying to prevent by first elucidating the pathological processes responsible so that effective therapies might be developed.

### *Immune Response*

Humans are protected from foreign pathogens, both bacterial and viral, by the immune system. There are two systems of immunity – innate, or natural immunity, and acquired, also known as adaptive or specific immunity. Essentially, what distinguishes the two types of immunity is their respective way of recognizing microorganisms (Fearon and Locksley, 1996). Innate immunity utilizes encoded proteins to recognize potentially harmful substances. For example, macrophages have a receptor for lipopolysaccharide (LPS), a component of Gram-negative bacterial cell membranes, which signals the presence of infection by eliciting cytokine synthesis. Some such cytokines are interleukin-1 (IL-1), IL-6, IL-12, and tumor necrosis factor (TNF). The function of these cytokines is to elicit immune response mechanisms such as development of helper T cells and stimulation of macrophage activity (Fearon and Locksley, 1996). The innate immune response is mediated by various cells types such as neutrophils, macrophages, and dendritic cells (Janeway et al., 1999a). Essentially the innate response is able to react immediately and destroy pathogens upon detection, whereas the acquired immune response requires time to plan a course of attack. Apparently, there are innate receptors present on all cells that are specific for certain types of pathogens, and these receptors allow for the immediacy of the innate immune response (Janeway, 1992).

Acquired immunity, on the other hand, comprises lymphocytes and is very adaptable (Fearon and Locksley, 1996). Adaptive (acquired) immunity is mediated by T-lymphocytes (T cells) and B-lymphocytes (B cells), each of which produces T cell receptors and B cell receptors respectively, which recognize foreign and self-antigens. These receptors are known to be part of the immunoglobulin family of proteins. As a result of extensive recombination of the genes that code for these receptors, each cell exhibits a structurally unique receptor. This recombination of genes takes place during early development and also results in production of T cells and B cells that express specific receptors capable of recognizing a wide range of both foreign and self-antigens (Medzhitov and Janeway, 2000). In 1959 Burnet mapped out the theory of clonal selectivity, which still serves to explain the notion of autoimmunity - how individuals are protected from self-tissue attack by the immune system. According to this theory, at the time of T cell differentiation in the thymus, autoreactive T cells are deleted. This process results in a wide range of T cells and B cells that recognize foreign but not self-antigens in the mature individual (Burnet, 1970). Furthermore, a T cell or B cell specific for an antigen that becomes activated by that antigen will undergo proliferation in order to produce sufficient numbers of cells necessary for an adequate response. This process is referred to as clonal expansion and results in the output of effector cells that respond directly against the pathogen that stimulated both their production and the production of memory cells. Memory cells allow for a more rapid response in the event of subsequent exposure. Memory cells are lymphocytes, and their production serves as the foundation for both active and passive immunity. Active immunity occurs in response to infection of pathogens whereas passive immunity occurs in response to antigens from inactivated pathogens following vaccination (Picker and Siegelman, 1999). Although it is clear that acquired immunity provides the body with powerful long-term immunity

and is highly antigen specific, acquired immunity is ineffective until memory and effector lymphocytes are produced. This process can take up to two weeks, making innate immunity vital in that it holds the invading pathogen in check, thus giving the acquired immune response time to gather forces for attack (Janeway et al., 1999b, c).

### *Etiology of MS*

MS is considered an autoimmune disease because, in individuals suffering from MS, the immune system attacks self-antigens in the body's CNS. It is this self-attack that causes neuropathology. In an effort to explain the development of autoimmunity, there are two primary theories that will be discussed here: the molecular mimicry theory and the clonal selection theory (Rouse and Deshpande, 2002; Burnet, 1959). Molecular mimicry is the idea that an immune response can be elicited by viral antigens that maintain sufficient molecular similarity to self-antigens. The reason that autoimmune diseases do not develop under normal circumstances is that during development, lymphocytes that have high affinity receptors for self antigens are deactivated (Rouse and Deshpande, 2002). Although autoreactive lymphocytes are still present, they are not activated in normal healthy individuals. Also, it has been shown that lymphocyte receptors on T cells might not be as specific as once thought. In effect, several epitopes might have the capability of reacting with a single receptor due to this degeneration of T cell recognition specificity. An epitope is the surface portion of an antigen. It is capable of both eliciting an immune response and of combining with the antibody produced to counter that response (Houghton Mifflin, 2002). In essence, once an antigen has exacted a response, the expansion of T cell clones could react with other epitopes not excluding self proteins presented by self major histocompatibility complex (MHC) (Rouse and Deshpande, 2002). Although the specific cause of MS is not known, many viruses could contribute its pathogenesis. This is



considered a possibility by some based on observations that relapses of symptoms in those suffering from MS are often preceded by microbial infections (Martin et al., 2001). In short, viral infections could initiate and cause expression of autoimmune disease when they result in a cross-reaction between host autoreactive T cells and the foreign agents (Rouse and Deshpande, 2002). The invading virus activates myelin-specific T cells by molecular mimicry (Martin et al., 2001).

As mentioned earlier, lymphocytes that have high-affinity receptors for self antigens are eliminated during development. This notion is the clonal selection theory of acquired immunity (Burnet, 1959). If, as the clonal selection theory (CST) holds, all antigen receptors that might recognize self-antigens must be purged, then autoimmunity is technically not possible. Regardless, autoimmune diseases do occur, often in the aftermath of infection. Keeping true to the CST, receptors are deleted that recognize self-antigens; however, an infectious agent is capable of disregarding the deletions and instead presenting epitopes that “mimic” host epitopes. So, while the CST keeps the immune system from stimulating self-recognition, “pseudo-self recognition” can occur. Accordingly, the clonal selection and molecular mimicry theories can coexist (Cohen, 2000).

There are several contributing factors to the development of MS and other autoimmune diseases (AID) such as environment, genetics, and hormones. It is thought that environmental factors play a role in triggering autoimmune disease even in individuals genetically predisposed to AID. According to Davidson and Diamond (2001), the importance of environmental triggers has become evident through studies of populations that are genetically similar, but because they live in different locations, are exposed to differing environmental conditions. Specific gene mutations have been shown to correlate to several autoimmune diseases. An example is found in comparing the rate of AID in monozygotic and dizygotic twins (Davidson and Diamond, 2001).



Specifically with MS, monozygotic twins have much higher concordance of the disease. So too, the disease is much more common in biologically related family members than in adopted family members with no biological relation (Noseworthy, 1999). It should be noted that while there are some autoimmune diseases that result from single gene mutations, the majority are associated with multiple gene mutations (Wucherpfennig, 2001).

Furthermore, hormonal control is thought to contribute to AID development. Multiple sclerosis occurs more often in females than males; in fact, this holds true for many other autoimmune diseases. While the mechanisms responsible for this gender difference are unclear, several studies have suggested that the disproportion is due to the influence of sex steroids on onset and exacerbations of MS (Drew and Chavis, 2000). It has been observed that steroidal changes that occur during gestation affect symptoms of MS. In short, during the third trimester of pregnancy there often is a general remission of symptoms while the postpartum period is marked by exacerbations (Confavreux et al., 1998).

Since the 1970s, mitoxantrone has been used to treat various non-lymphocyte leukemias as well as both prostate and breast cancer (Neuhaus et al., 2006). However, in October of 2000, the FDA approved the agent for treatment of severe forms of relapsing-remitting MS and secondary progressive MS (Koeller and Eble, 1988; Jain, 2000). Because MS is thought to be an autoimmune disease, immunosuppressants such as mitoxantrone might slow the progression of demyelination. Mitoxantrone is a powerful immunosuppressive drug that targets proliferating immune cells such as T and B lymphocytes and macrophages (Neuhaus et al., 2006). Chemically, mitoxantrone, 1,4-Dihydroxy-5,8-bis-[[2-[(2-hydroxyethyl)-amino]-ethyl]-amino]-anthraquinone dihydrochloride,  $C_{22}H_{28}N_4O_6 \cdot 2HCl$ , is a synthetic anthracenedione derivative with a molecular mass of 517.4 Da (Neuhaus et al., 2006) (Figure 1). Mitoxantrone is a topoisomerase II inhibitor

(Koeller and Eble, 1988), and this interaction results in single- and double- strand breaks in DNA by ligating the DNA at specific sites in the course of synthesis (Neuhaus et al., 2006). What occurs is an arrest at an important step in synthesis and is believed to be caused by mitoxantrone stabilizing the cleavable complex of topoisomerase II (Osheroff et al., 1994). This halt in cell cycle progression leads to an accumulation of cells in the G2 phase, and this activity of mitoxantrone has been demonstrated in human fibroblast cell lines (Fox and Smith, 1990).

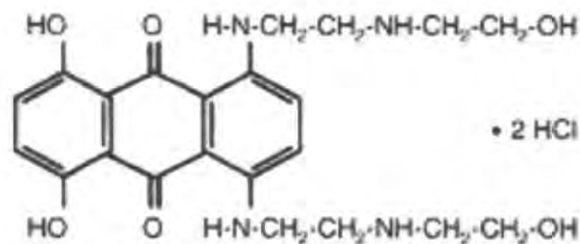


Figure 1. Structure of mitoxantrone.

Pharmacokinetically, mitoxantrone is 78% bound to plasma proteins, has a plasma half life somewhere between 25 and 215 hours, and is eliminated by both the liver and kidneys. However, from the liver and kidneys collectively, less than 35% was recovered within five days after administration. More so, the agent has been shown to remain in cancer patients for close to nine months. Unfortunately, studies on the ability of mitoxantrone to penetrate the blood-brain barrier have shown contradiction (Neuhaus et al., 2006). Extent of treatment is cumulative dose of 140 mg/m<sup>2</sup>, or a time period of 2 to 3 years (Neuhaus et al., 2005). These restrictions on mitoxantrone treatment time and dosage are due to the drug's association with cardiotoxicity, specifically congestive heart failure. The mechanisms by which mitoxantrone modulates multiple sclerosis have not been elucidated, and it is our hope that clarification of these pathways will lead to safer, more effective therapies for individuals living with this disease.

### *Role of microglia in MS*

Central to the pathology of MS is the MS lesion and subsequent demyelination. Myelin destruction occurs amidst an inflammatory reaction consistent of T cells, B cells, macrophages, and widespread microglial/macrophage activation (Bruck, 2005). Therefore, not only do autoreactive T cells play a role in MS pathology, but activated microglia are also thought to take part. Microglia are the primary cellular component of the endogenous CNS immune/inflammatory defense system (Ransohoff, 1999). These cells are found near endothelial cells, sporadically in the parenchyma, and in the perivenular regions (Sriram and Rodriguez, 1997). Characteristic of microglia is their fast response to inflammation, injury, infection, and neurodegeneration (Benveniste, 1997). In order for T cells to recognize antigens, these antigens must be presented and this process is carried out by antigen presenting cells (APCs). Microglial cells can function as antigen-presenting cells and produce and respond to a wide variety of cytokines. However, it has been established that microglia in and surrounding MS lesions become activated, and upon doing so, undergo a morphological change into macrophages (Ransohoff, 1999). They can impair blood-brain barrier (BBB) function, mediate phagocytic events, and damage oligodendrocytes, the cells that produce myelin in the central nervous system (Benveniste, 1997). The MS inflammatory reaction is linked to an up-regulation of various cytokines such as interleukin-6 (IL-6), tumor necrosis factor-alpha (TNF- $\alpha$ ), and chemokines such as mouse monocyte chemotactic protein-1 (MCP-1). Cytokines are proteins that are made by cells that affect the behavior of other cells. Cytokines made by lymphocytes are often referred to as interleukins (IL) or lymphokines (Janeway and Travers, 1994). Chemokines are simply small cytokines and are involved in migration and activation of cells, specifically phagocytic cells and lymphocytes (Janeway and Travers, 1994). The activated microglia produce these

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Figure 2 represents a schematic model of CNS demyelination. As seen, the process is initiated by microglial activating factors. Activated microglia secrete cytokines, organic molecules, excitatory amino acids, and chemokines that sustain inflammation and demyelination. (HSP = heat shock protein; EAA = excitatory amino acid.)

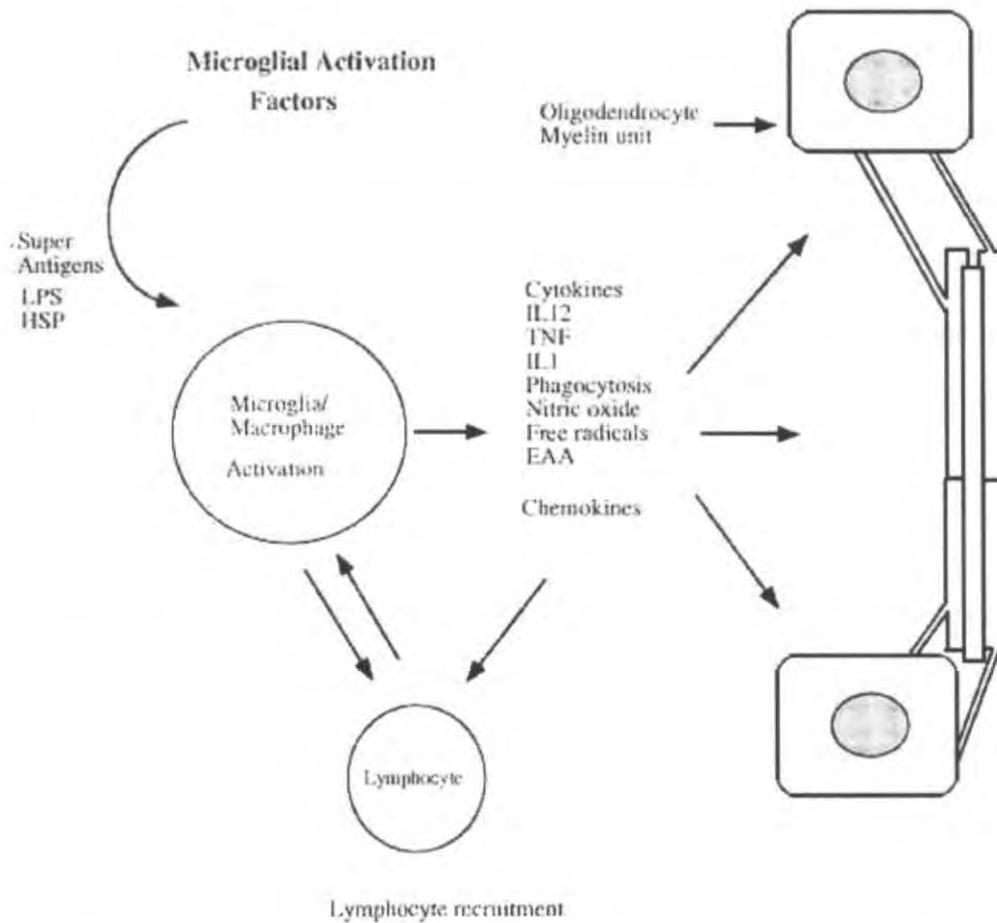


Figure 2. Schematic model of CNS demyelination  
 From: Sriram: Neurology, Volume 48(2).February 1997.464-470  
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### *Microglia: effects on T cell phenotype*

In order to evaluate the effects of microglia on T cell phenotype, specific mouse strains are induced with experimental autoimmune encephalomyelitis (EAE). EAE is a neurodegenerative disease specifically occurring in rodents and serves as an animal model for MS (Benveniste, 1997). EAE is induced in rodents by the adoptive transfer of CD4<sup>+</sup> T cells that are specific for myelin antigens (Drew, 2005). T-helper 1 (Th1) and T-helper 2 (Th2) cells are the two types of CD4<sup>+</sup> T cells. Each type produces different types of cytokines. Th1 cells produce and release IL-1, IL-2, TNF- $\alpha$ , lymphotoxin (LT), and IFN- $\gamma$ , whereas Th2 cells produce and release IL-4, IL-10, IFN- $\beta$ , and TGF- $\beta$ , which appear to be linked to disease remission (Benveniste, 1997). Secondly, Th1 cell response is coupled with cell-mediated immunity and is responsible for delayed-type hypersensitivity, while Th2 cell reaction is associated with humoral immunity, stimulation of allergic response mechanisms, and increased production of antibodies (Drew, 2005).

### *Microglia: antigen presentation*

Lymphocytes are the primary components of the immune response, but they rely heavily on phagocytic cells of the monocyte-macrophage system for presentation of antigen to T lymphocytes. In the case of autoimmune disease, these APCs present self-peptides to T cells resulting in pathology. This process is referred to as a failure of self-tolerance (Young and Heath, 2000). It is important to note that both the innate component, as well as the acquired component of the immune system, play a role in antigen presentation. Cells derived from monocytes, such as macrophages and microglia, are representative of the innate component and are collectively referred to as "professional APC." Equally, cells of the adaptive component are CD4<sup>+</sup> T-helper

cells, CD8<sup>+</sup> T-cells, and B cells. This class is referred to as major histocompatibility complex (MHC) (Drew, 2005).

## **Materials**

### *Mitoxantrone*

Mitoxantrone, 1,4-Dihydroxy-5,8-bis-[[2-[(2-hydroxyethyl)-amino]-ethyl]-amino]-anthraquinone dihydrochloride, (C<sub>22</sub>H<sub>28</sub>N<sub>4</sub>O<sub>6</sub> • 2HCl) was prepared in dimethyl sulfoxide (DMSO) to a final stock concentration of 20 mM (Drew).

### *Lipopolysaccharide (LPS)*

Lipopolysaccharide (LPS) was prepared in sterile phosphate buffer saline (PBS) to a stock concentration of 500µg/ml.

## **Methods**

### *Cell Culture*

The N9 microglial cell line was derived from mouse cerebrum and immortalized by transfection with the v-myc oncogene (Righi et al., 1989; Corradin et al., 1993). N9 cells were cultured at 37° C and 5.0% CO<sub>2</sub> in Eagle's minimum essential medium with Earle's salts (Mediatech Cellgro, Herndon, VA) containing a final concentration of 10% fetal bovine serum (FBS) and 1.38mM L-glutamine (Sigma, St. Louis, MO). The N9 cell line was provided as a gift to Lori Hensley, Ph.D. (Ouachita Baptist University, Arkadelphia, AR, USA) by Paul Drew, Ph.D. (University of Arkansas for Medical Sciences, Little Rock, AR, USA).



### *MTT Assay*

Cell viability was measured with an MTT (3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide) assay, which measures mitochondrial activity. MTT solution was prepared by diluting MTT 1:50 with culture medium. Cell culture media was removed and replaced with 1ml MTT solution and incubated at 37° C for one hour. MTT solution was removed and cells were lysed by adding 1ml DMSO/well. Plates were rocked for 30 minutes and read on a plate reader at 570nm.

### *Nitrite Assay*

For 100ml Gries reagent, 1g of sulfanilamide, 0.1g N(1-naphthyl)ethylenediamine (C<sub>12</sub>H<sub>14</sub>N<sub>2</sub> 2HCl), 2.5ml phosphoric acid, and 97.5ml H<sub>2</sub>O were added together. Cells were plated in a 6-well plate at  $6.4 \times 10^5$  cells/well and incubated for 24 hours before treatment with mitoxantrone. Cells were then incubated 4 hours before inducing activation of N9 microglial cells with LPS. Media were harvested from wells after 24 hours, and 50µl of each sample was added to a 96-well plate with 50µl Gries reagent and read on a plate reader at 550nm.

### *IL-6 ELISA Assay*

IL-6 production by microglia was determined according to manufacturer's recommendations (BD Biosciences-PharMingen, San Diego, CA). For analysis of IL-6, microwells were coated with 100µl per well of Capture Antibody diluted in Coating Buffer. The plate was sealed and incubated overnight at 4° C. The wells were aspirated and washed 3 times with 300µl/well of wash buffer. The plates were then blocked with 200µl/well of Assay Diluent. The plate was then incubated at room temperature for 1 hour. The wells were again aspirated and washed 3 times with 300µl/well of wash buffer. Standard and sample dilutions in Assay Diluent were prepared. 100µl of each standard, sample, and control were pipetted into appropriate wells.

The plate was sealed and incubated for 2 hours at room temperature. The wells were again aspirated and washed, but with a total of 5 washes. 100µl of Working Detector (Detection Antibody + SAV-HRP reagent) were added to each well, and the plate was sealed and incubated for 1 hour at room temperature. The wells were then washed again, but with 7 total washes. 100µl of Substrate Solution were then added to each well. Next, the plate was left to incubate unsealed in the dark for 30 minutes at room temperature. 50µl of Stop Solution were then added to each well. Finally, the absorbance was read at 450nm within 30 minutes of stopping reaction.

#### *IL-1β ELISA Assay*

IL-1β production by microglia was determined according to manufacturer's recommendations (BD Biosciences-PharMingen, San Diego, CA). For analysis of IL-1β, microwells were coated with 100µl per well of Capture Antibody diluted in Coating Buffer. The plate was sealed and incubated overnight at 4° C. The wells were aspirated and washed 3 times with 300µl/well of wash buffer. The plates were then blocked with 200µl/well of Assay Diluent. The plate was then incubated at room temperature for 1 hour. The wells were again aspirated and washed 3 times with 300µl/well of wash buffer. Standard and sample dilutions in Assay Diluent were prepared. 100µl of each standard, sample, and control were pipetted into appropriate wells. The plate was sealed and incubated for 2 hours at room temperature. The wells were again aspirated and washed, but with a total of 5 washes. 100µl of Detection Antibody diluted in Assay Diluent were added to each well. The plate was then sealed and incubated at room temperature for 1 hour. The wells were again washed, with a total of five washes. Next, 100µl of Enzyme Reagent diluted in Assay Diluent were added to each well. The plate was sealed and incubated for 30 minutes at room temperature. The wells were then washed with a total of 7 washes, followed by the addition of 100µl of Substrate Solution to each well. The plate was then

incubated without plate sealer for 30 minutes at room temperature in the dark. 50µl of Stop Solution was then added to each well. The absorbance was read at 450nm within 30 minutes of stopping reaction.

#### *MCP-1 ELISA Assay*

MCP-1 production by microglia was determined according to manufacturer's recommendations (BD Biosciences-PharMingen, San Diego, CA). For analysis of MCP-1, microwells were coated with 100µl per well of Capture Antibody diluted in Coating Buffer. The plate was sealed and incubated overnight at 4° C. The wells were aspirated and washed 3 times with 300µl/well of wash buffer. The plates were then blocked with 200µl/well of Assay Diluent. The plate was then incubated at room temperature for 1 hour. The wells were again aspirated and washed 3 times with 300µl/well of wash buffer. Standard and sample dilutions in Assay Diluent were prepared. 100µl of each standard, sample, and control were pipetted into appropriate wells. The plate was sealed and incubated for 2 hours at room temperature. The wells were again aspirated and washed but with a total of 5 washes. 100µl of Working Detector were added to each well, and the plate was sealed and incubated for 1 hour at room temperature. The wells were then washed again but with 7 total washes. 100µl of Substrate Solution were then added to each well. Following this step, the plate was left to incubate unsealed in the dark for 30 minutes at room temperature. 50µl of Stop Solution were then added to each well. Finally, the absorbance was read at 450nm within 30 minutes of stopping the reaction.

## **Results**

### **The Effect of Mitoxantrone on the viability of N9 microglial cells induced by LPS**

Cell viability was measured with an MTT (3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide) assay, which measures mitochondrial activity. Cells were pre-treated for 4 hours with the indicated concentrations of the chemotherapy agent mitoxantrone. LPS was added as indicated and 24h later cell viability was determined. If the cells were living, mitochondria would convert the chemical and show purple color. If cells were dead, there would be no purple color. Our results indicated that mitoxantrone does not affect the viability of N9 microglial cells induced by LPS (Figure 3).

### **The Effects of Mitoxantrone on Nitrite Production in N9 Microglia**

Nitrite (NO), in large enough amounts, can be toxic to myelin producing oligodendrocytes. This assay was carried out to specifically determine the effect of mitoxantrone on NO production in N9 microglia cells. Cells were pre-treated for 4 hours with the indicated concentrations of the chemotherapy agent mitoxantrone. LPS was added as indicated, and 24h later the concentration of nitrite in the culture medium was determined. Our results indicate that mitoxantrone significantly inhibits LPS-induced production of nitrite in N9 microglial cells, as hypothesized (Figure 4). An MTT assay was performed to determine cell viability, and it was revealed that mitoxantrone does not affect N9 microglial viability. From these results, we can thus conclude that the LPS-induced inhibition of NO production by mitoxantrone is not a result of the compound's effects on viability.

### **The Effects of Mitoxantrone on IL-6 Production in N9 Microglia**

IL-6 is a cytokine produced by macrophages and has long-range effects that aid in host defense. IL-6 is an endogenous pyrogen, meaning it arises from an endogenous source and causes fever, which contributes to fighting infection. T cells and B cells augment antigen processing and thus increase the adaptive immune response (Janeway and Travers, 1994). In the event of an autoimmune disease, T cells attack self tissue “thinking” it is foreign.

Cells were pre-treated for 4 hours with the indicated concentrations of the chemotherapy agent mitoxantrone. LPS was added at a final concentration of .0626mg/ml as indicated and 24h later the concentration of IL-6 in the culture medium was determined. It was shown that mitoxantrone inhibited the LPS-induced production of IL-6 in N9 microglial cells. Cell viability was determined using an MTT assay, and it was shown that mitoxantrone does not reduce microglial cell viability when compared to LPS-treated cells. (See figure 5)

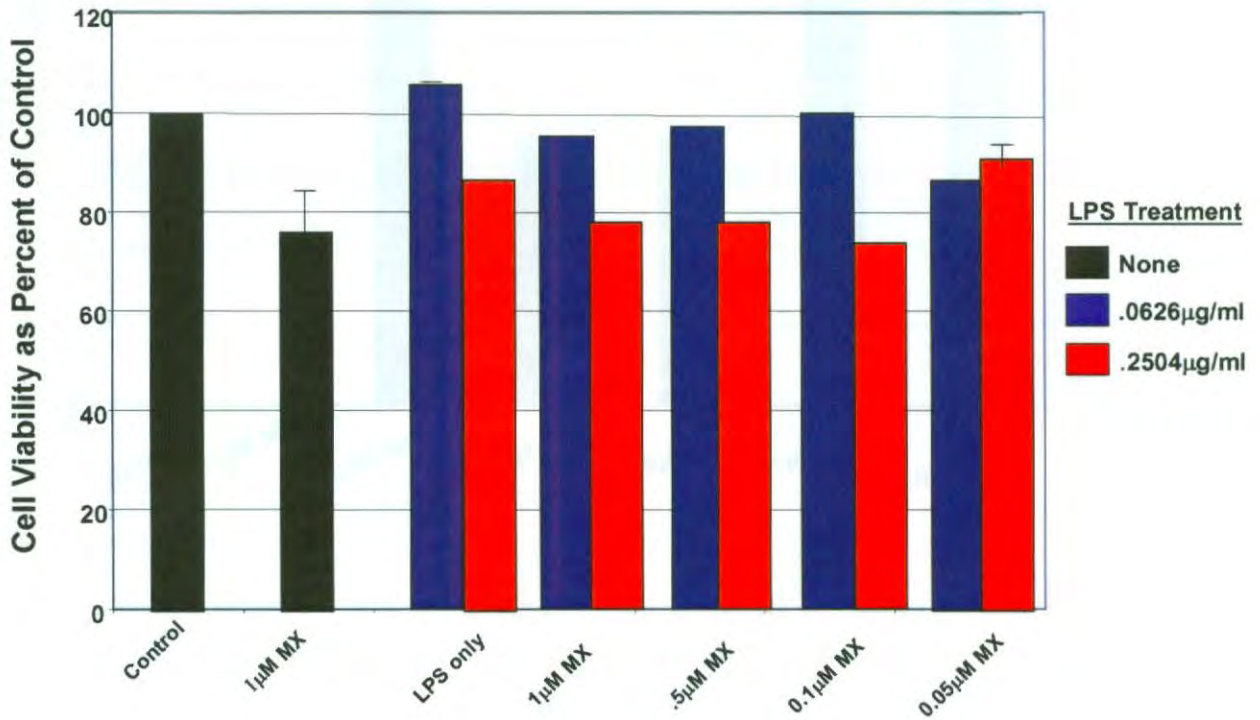
### **The Effects of Mitoxantrone on IL-1 $\beta$ Production in N9 Microglia**

Although the procedure to determine IL-1 $\beta$  (interleukin-1 $\beta$ ) production by microglia was carried out according to the manufacturer’s recommendations (BD Biosciences-PharMingen, San Diego, CA), the concentration of LPS was not sufficient to elicit LPS stimulation in the microglia cells. The concentration was adjusted in step-wise fashion, but even at a four-fold increase in LPS it was still not adequate to stimulate cytokine production.

### **The Effects of Mitoxantrone on MCP-1 production in N9 Microglia**

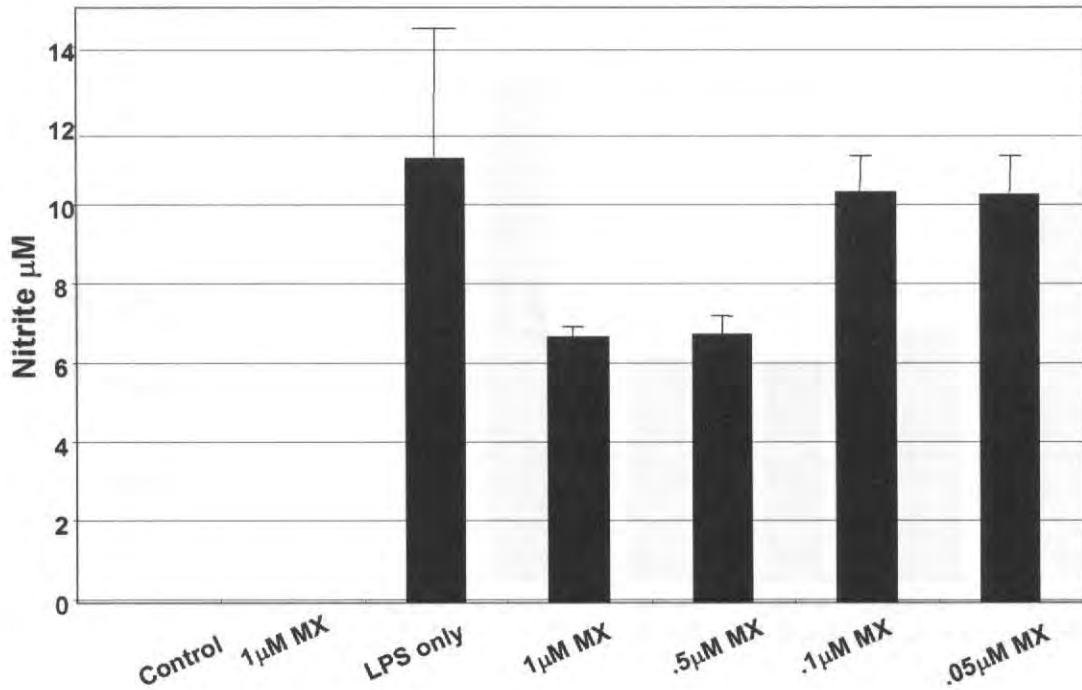
Although the procedure to determine MCP-1 (mouse monocyte chemotactic protein-1) production by microglia was carried out according to the manufacturer’s recommendations (BD Biosciences-PharMingen, San Diego, CA), the resulting data were outside the linear range of the

plate reader. Therefore, the medium was diluted to 1: 250, but results still remained outside linear range.



**Figure 3. Mitoxantrone does not affect the viability of N9 microglial cells induced by LPS.** Cells were pre-treated for 4 hours with the indicated concentrations of the chemotherapy agent mitoxantrone. LPS was added as indicated and 24h later cell viability was determined. Values represent the average for triplicate cultures and standard deviations are indicated.

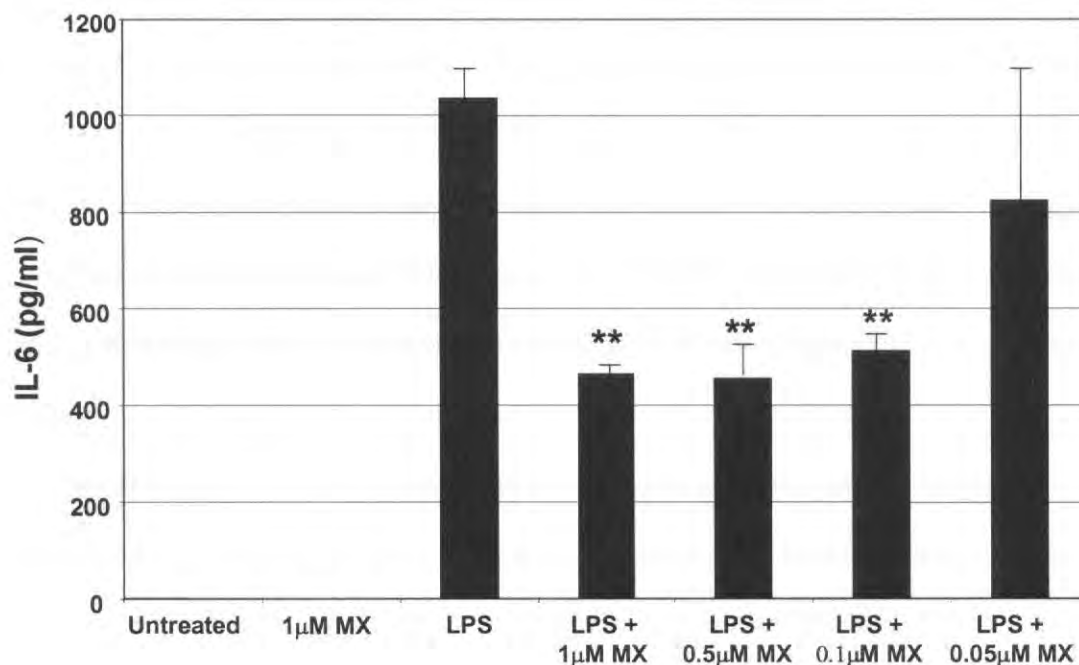




**Figure 4. Mitoxantrone inhibits LPS induction of nitrite in N9 microglia cells.**

Cells were pre-treated for 4 hours with the indicated concentrations of the chemotherapy agent mitoxantrone. LPS was added as indicated and 24h later the concentration of nitrite in the culture medium was determined.





\*\* indicates statistical significance as compared to LPS-treated

**Figure 5. Mitoxantrone inhibits LPS induction of IL-6 in N9 microglia cells.**

Cells were pre-treated for 4 hours with the indicated concentrations of the chemotherapy agent mitoxantrone. LPS was added at a final concentration of .0626mg/ml as indicated and 24h later the concentration of IL-6 in the culture medium was determined. Values represent the average for triplicate cultures and standard deviations are indicated.

## Discussion

In October of 2000, the FDA approved mitoxantrone for treatment of severe forms of relapsing-remitting MS and secondary progressive MS (Koeller and Eble, 1988; Jain, 2000). However, mitoxantrone has been used to treat various non-lymphocyte leukemias as well as prostate and breast cancer since the 1970s, (Neuhaus et al., 2006). MS is thought to be an autoimmune disease, and for this reason, immunosuppressants such as mitoxantrone might slow the progression of demyelination. Mitoxantrone is a powerful immunosuppressive drug that targets proliferating immune cells such as T and B lymphocytes and macrophages (Neuhaus et al., 2006).

Mitoxantrone has been shown to repress microglial activation as well as impede proliferation of lymphocytes induced by antigen. The pathway by which mitoxantrone might repress activation of microglia in autoimmune disease is mediated by a type of feedback loop between cytokines and T-helper cells (Drew, 2005). Activated microglia/macrophages present antigen via MHC molecules to CD4<sup>+</sup> T-helper cells. Subsequently, said T-cells become activated which then produce and release cytokines which in turn exacerbates the activation of the microglia/macrophages. These molecules, such as NO, TNF- $\alpha$ , and interleukins, released from activated microglia, can have an adverse effect on oligodendrocytes. Damaged oligodendrocytes result in demyelination of nerves which lead to the severe symptoms MS (Benveniste, 1997).

Our results indicate that mitoxantrone significantly inhibits LPS-induced production of nitrite in N9 microglial cells, as hypothesized. An MTT assay was performed to determine cell viability, and it was revealed that mitoxantrone does not affect N9 microglial viability. From these results, we can conclude that the LPS-induced inhibition of NO production by mitoxantrone is not a result of the compound's effects on viability. By reducing NO with

mitoxantrone, neurodegeneration could be significantly curtailed, implying an alleviation of MS symptoms and heightened viability of myelin-producing oligodendrocytes. Moreover, mitoxantrone has also been shown through our studies to inhibit interleukin-6 (IL-6), an inflammatory cytokine in microglial cells. As evidenced in Figure 4, IL-6 was inhibited in microglial cells by mitoxantrone. The implication is that cytotoxicity of oligodendrocytes is decreased and thus neurodegeneration moderated.

Although the procedure to determine IL-1 $\beta$  production by microglia was carried out according to the manufacturer's recommendations (BD Biosciences-PharMingen, San Diego, CA), the concentration of LPS was not sufficient to elicit LPS stimulation in the microglia cells. The concentration was adjusted in step-wise fashion, even at a four-fold increase in LPS, but it was still not adequate to stimulate cytokine production. In the future this assay will continue to be run at varied concentrations of LPS in effort to determine the concentration at which LPS stimulation occurs. Similarly, although the procedure to determine MCP-1 (mouse monocyte chemotactic protein-1) production by microglia was carried out according to the manufacturer's recommendations (BD Biosciences-PharMingen, San Diego, CA), the resulting data were outside the linear range of the plate reader. Therefore, the medium was diluted to 1: 250, but results still remained outside linear range. This assay will continue to be run at varied dilutions of media samples until samples fall into the linear range.

In conclusion, mitoxantrone was seen to maintain an inhibitory effect on LPS-induced N9 microglial cell activation. Specifically, these effects include the marked decrease of proinflammatory molecules such as NO and IL-6. As it has been observed that these molecules mediate pathogenesis of the neurodegenerative disease multiple sclerosis, the repression of activated microglia could potentially lead to an increase in oligodendrocyte viability and, thus,

lessened severity of multiple sclerosis. Our lab plans to investigate a new agent, ajulemic acid, using the same experiments described here. Our hope is that it will modulate microglial activation in a similar manner and may one day provide a safe and more effective treatment option for those living with MS.

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