Published in final edited form as:

Brain Res. 2009 August 25; 1286: 174–184. doi:10.1016/j.brainres.2009.06.007.

Attenuation of oxidative stress, inflammation and apoptosis by minocycline prevents retrovirus-induced neurodegeneration in mice

Xianghong Kuang^a, Virginia L. Scofield^a, Mingshan Yan^a, George Stoica^b, Na Liu^a, and Paul K.Y Wong^{a,*}

^aDepartment of Carcinogenesis, The University of Texas, M.D. Anderson Cancer Center, Science Park-Research Division, Smithville, TX 78957

^bDepartment of Pathobiology, Texas A&M University College of Veterinary Medicine, College Station, TX 77843.

Abstract

The *ts1* mutant of the Moloney murine leukemia virus (MoMuLV) causes neurodegeneration in infected mice that resembles HIV-associated dementia. We have shown previously that *ts1* infects glial cells in the brain, but not neurons. The most likely mechanism for *ts1*-mediated neurodegeneration is loss of glial redox support and glial cell toxicity to neurons. Minocycline has been shown to have neuroprotective effects in various models of neurodegeneration. This study was designed to determine whether and how minocycline prevents paralysis and death in *ts1*-infected mice. We show here that minocycline delays neurodegeneration in *ts1*-infected mice, and that it prevents death of cultured astrocyte infected by *ts1* through attenuating oxidative stress, inflammation and apoptosis. Although minocycline reduces virus titers in the CNS of infected mice, it does not affect virus titers in infected mice thymi, spleens or infected C1 astrocytes. In addition, minocycline prevents death of primary neurons when they are cocultured with *ts1*-infected astrocytes, through mechanisms involving both inhibition of oxidative stress and upregulation of the transcription factor NF-E2-related factor 2 (Nrf2), which confers the cell the antioxidant defense. We conclude that minocycline delays retrovirus *ts1*-induced neurodegeneration involving antioxidant, anti-inflammation and anti-apoptotic mechanisms.

Keywords

Moloney murine leukemia virus -*ts1*; Minocycline; Oxidative stress; Inflammation; Apoptosis; Nrf2

1. Introduction

ts1, a neuropathogenic mutant of Moloney murine leukemia virus (MoMuLV), induces a progressive neurodegenerative disease in susceptible strains of mice (Stoica et al., 1993;

© 2009 Elsevier B.V. All rights reserved.

*Corresponding Author: Department of Carcinogenesis, University of Texas, M.D. Anderson Cancer Center, Science Park-Research Division, 1808 Park Road 1-C, P.O. Box 389, Smithville, Texas 78957 Tel: 512-237-9456, Fax: 512-237-2444; e-mail: pkwong@mdanderson.org (PK. Wong).

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

Wong et al., 1991; Wong, 1990). This neurodegenerative disease is morphologically manifested as spongiform encephalomyelopathy, and clinically manifested as hindlimb paralysis and wasting, eventually leading to death of infected animals (Wong et al., 1992). The *ts1* virus has a mutation that results in a substitution of isoleucine for valine at position 25 in the viral envelope precursor protein gPr80^{env} (Szurek et al., 1990). This alteration makes the virus cytopathic to astrocytes, due to abnormal accumulation of uncleaved gPr80^{env}. In turn, the infected astrocytes may induce motor neuron loss in infected mice (Stoica et al., 2000; Wong and Yuen, 1994; Wong PKY, 1992).

Although neurodegeneration is the end result of central nervous system (CNS) damage after *ts1* infection, the virus replicates in astrocytes, microglia, oligodendrocytes and endothelial cells, but not in neurons (Stoica et al., 1993; Wong and Yuen, 1994). In this respect, the *ts1* induced neurodegenerative syndrome is to some extent similar to the neuropathology of HIV-1-associated dementia (HAD) (Clark et al., 2001; Gonzalez-Scarano, 1995; Mollace et al., 2001; Stoica et al., 1993). *ts1*- and HIV-1-induced neurodegeneration also share pathological features with other nonviral neurological diseases, such as Parkinson's disease (PD), Alzheimer's disease (AD) and amyotrophic lateral sclerosis (ALS). These conditions resemble the *ts1* syndrome in that they all involve oxidative stress, inflammation and apoptosis (Infante-Duarte et al., 2008; Liu et al., 2004; Qiang et al., 2004; Reynolds et al., 2007).

Minocycline is a semisynthetic second-generation tetracycline compound that effectively crosses the blood-brain barrier (Yong et al., 2004). This drug has been shown to have neuroprotective activity in primates infected with Simian immunodeficiency virus (SIV) (Zink et al., 2005). It also protects the CNS in ischemia-perfusion injury (Yrjanheikki et al., 1999), Huntington's disease (Chen et al., 2000), PD (Du et al., 2001), AD (Noble et al., 2009) and ALS (Zhu et al., 2002). The mechanisms involved in neuroprotection by minocycline appear to be distinct from its antimicrobial activities (Tikka et al., 2001).

Recent work has shown that minocycline is a potent antioxidant, with radical scavenging activities (Kraus et al., 2005; Morimoto et al., 2005). Both laboratory and clinical studies have also shown that minocycline has broad-spectrum anti-inflammatory properties (Sapadin and Fleischmajer, 2006) that are manifested via inhibition of cyclooxygenase-2 (COX-2) (Yrjanheikki et al., 1999), nuclear factor- κ B (NF- κ B) (Nikodemova et al., 2006; Si et al., 2004) and activation of microglia (Dheen et al., 2007; Tikka et al., 2001; Zink et al., 2005). Minocycline also exerts anti-apoptotic effects (Choi et al., 2007; Stirling et al., 2004), via both caspase-dependent and caspase-independent pathways (Stirling et al., 2005).

In this study, we first demonstrated that minocycline significantly delays *ts1*-induced neurodegeneration in infected mice. We then identified mechanisms underlying the neuroprotective effects of minocycline on cultured astrocytes, and on primary neurons that were either cocultured with *ts1*-infected astrocytes, or exposed to spent medium from these astrocyte cultures. We conclude that neuroprotection by minocycline involves direct radical scavenging and upregulation of Nrf2-mediated antioxidant defenses. Its neuroprotection also involves anti-inflammatory and anti-apoptotic mechanisms.

2. Results

2.1 Minocycline not only delays paralysis and death, but also attenuates astrogliosis lesion in *ts1*-infected FVB/N mice

The paralysis/survival curves in Figure 1A show that untreated *ts1*-infected (*ts1*-only) mice became paralyzed at around 35 dpi, while *ts1*-infected minocycline-treated (*ts1*-mino) mice lived longer, and became paralyzed much later ($p < 0.001$). Brainstem and spinal cord tissues

were prepared from uninfected, *ts1*-only and *ts1*-mino mice, and stained with hematoxylin and eosin (H&E). The sections from uninfected mice showed normal neuropil in the brainstem, while those from *ts1*-only mice contained many spongiform lesions, with large vacuoles. By contrast, sections from *ts1*-mino mice had nearly normal cellular organization, with a few small vacuoles, but without discrete spongiform foci (Fig.1B). When we stained the *ts1*-only brainstem tissues with astrocyte marker GFAP, we observed an increased in number and brightness of GFAP-expressing astrocytes when compared with uninfected brainstem sections, which indicates astrogliosis was induced in *ts1*-only brainstems. However, nearly normal GFAP expression is displayed in *ts1*-mino brain sections (Fig. 1C), indicating that astrogliosis lesion is prevented by minocycline treatment.

2.2 Minocycline prevents cell death in *ts1*-infected C1 cells

To assess the effects of minocycline on CNS cells, we examined its cytoprotective effect for *ts1* -infected C1 astrocytes. Figure 2 shows that at 48 and 72 h, *ts1*-only C1 cells underwent significant cell death, when compared to uninfected cells. When minocycline was added to these cultures, however, many of the infected cells remained alive (Fig. 2).

2.3 Minocycline reduces viral titer in the CNS

To determine whether minocycline reduces virus titers in the CNS, brainstems and spinal cords were removed from *ts1*-only and *ts1*-mino mice at 30 dpi, and subjected to the viral titer protocol described in Experimental procedures. Figure 3A shows that *ts1*-mino CNS tissues had reduced viral titers, relative to those of *ts1*-only mice. To determine whether reduction of virus titers by minocycline is specific to the CNS, we also collected thymi and spleens from *ts1*-only and *ts1*-mino mice at 30 dpi, and measured viral titers in these organs. Notably, we found that no differences in virus titers in thymi and spleens from *ts1*-mino mice vs. *ts1*-only mice (Fig. 3A). These data show that minocycline reduces virus titer in CNS tissues of *ts1*-mino mice, but not in thymi and spleens. To determine whether minocycline has a direct antiviral effect, we assessed the effects of minocycline on virus replication in cultured C1 astrocytes. Surprisingly, virus titers in the culture medium of *ts1*-mino cells were similar to those of *ts1*-only cells (Fig. 3B). These data indicate that minocycline at the concentration tested in this study do not directly affect virus replication in vitro, although minocycline prevents cell death in *ts1* -infected C1 cells. Thus virus replication is not the primary target for the cytoprotection by minocycline in cultured astrocytes.

2.4 Minocycline inhibits oxidative stress in *ts1*-infected-C1 astrocytes, through direct radical scavenging activity and upregulating Nrf2-mediated antioxidant defense

To find out whether protection of C1 cells by minocycline involves direct antioxidant activity, we treated uninfected C1 cells with 100 μ M of hydrogen peroxide (H_2O_2) for 1 h, and then measured their relative ROS levels using CM- H_2 DCFDA fluorescence. Figure 4A shows that ROS levels increased about 4-fold in H_2O_2 -treated cells in comparison with untreated cells. When increasing doses of minocycline were added immediately after addition of H_2O_2 , however, amounts of intracellular ROS were reduced in a minocycline dose-dependent fashion. This observation shows that minocycline has direct and rapid antioxidant effects in C1 cells.

To determine whether minocycline treatment has similar antioxidant effects on *ts1*-infected C1 cells, we replaced H_2O_2 treatment with *ts1* infection, by adding virus to the cell cultures, again following their ROS levels with CM- H_2 DCFDA. Figure 4B shows that ROS levels are significantly increased in *ts1*-only C1 cells relative to uninfected control cultures, but that treatment of the infected cells with 25 and 50 μ M minocycline decreased ROS levels in a minocycline dose-dependent manner. Figure 4C shows that adducts of the oxidative marker

malondialdehyde (MDA), the end product of lipid peroxidation, are also increased in *ts1*-only C1 cells relative to uninfected cultures, but not in *ts1*-mino cells.

Finally, we asked whether the antioxidant effects of minocycline also involve activation of endogenous antioxidant defenses pathways in *ts1*-infected C1 cells. Figure 4D shows that amounts of the antioxidant defense-related protein Nrf2 are reduced in *ts1*-only C1 cells, relative to uninfected cultures, while levels of Nrf2 are upregulated in *ts1*-mino cells. Together these data show that minocycline provides antioxidant protection to H₂O₂-treated or *ts1*-infected C1 cells, and that the mechanisms for this defense include both radical scavenging and Nrf2 related antioxidant defense.

2.5 Minocycline reduces markers of inflammation in the *ts1*-infected brainstem

In *ts1*-infected astrocytes or animals, NF- κ B and COX-2 are upregulated, while the NF- κ B inhibitor I κ B α is reduced in *ts1* infected astrocytes or animals (Kim et al., 2001; Kim et al., 2005). To determine whether minocycline treatment regulate levels of NF- κ B and COX-2, cell lysates were prepared from cultured uninfected cells, *ts1*-only or *ts1*-mino C1 cells and were subjected to Western blotting for levels of I κ B α , nuclear NF- κ B p65 and COX-2. Figure 5A shows that levels of nuclear NF- κ B p65 were increased in *ts1*-only C1 cells when compared to levels in uninfected C1 cells, while amounts of the NF- κ B inhibitor I κ B α are downregulated in *ts1*-only C1 cells, in association with translocation of NF- κ B from the cytoplasm to the nuclei of the cells. These data are consistent with the report from this laboratory (Kim et al., 2001). In *ts1*-mino C1 cells, however, levels of nuclear NF- κ B p65 are reduced, relative to the elevations in *ts1*-only cells, while amounts of I κ B α are markedly upregulated. These results are also in agreement with a report from other group indicating that minocycline inhibits the degradation of I κ B α in microglia (Nikodemova et al., 2006). Our data also show that COX-2 levels are elevated in cultured *ts1*-only C1 cells, but that this elevation is reduced in *ts1*-mino C1 cells.

To determine whether in vivo minocycline protection of the *ts1*-infected CNS involves reduction of inflammation in the tissue, we stained paraffin sections of brainstem from *ts1*-only vs. *ts1*-mino mice, sacrificed at 30 dpi, for the inflammation marker COX-2. We have shown previously that COX-2 levels are upregulated in the *ts1*-only brainstem (Kim et al., 2005). The same result is evident in the anti-COX-2-stained *ts1*-only brainstem sections (Fig 5B). Strong COX-2 immunoactivity (red), is especially evident in astrocytes (brown color). By contrast, the *ts1*-mino brainstem sections had no spongiform lesions, and showed weak COX-2 staining when compared to *ts1*-only brainstem sections. Since *ts1* -infected CNS showed activation of microglia (Stoica et al., 1993; Zachary et al., 1997), and that minocycline inhibited microglia activation in various neurodegeneration models (Dheen et al., 2007; Zachary et al., 1997; Zink et al., 2005), we ask whether minocycline has inhibition effects on microglia activation in *ts1* -infected brainstems. To address this question, we performed immunofluorescence staining to examine expression of CD68, a marker of activated microglia or macrophage (Zink et al., 2005), using frozen brainstem sections. Figure 5C shows that strong immunoactivity of CD68 is evident on *ts1*-only brainstem sections, especially near the area where spongiform lesion was severe. By contrast, CD68 positive cell is not visible on either *ts1*-mino or uninfected brain sections. These data indicate that minocycline attenuates inflammatory responses induced by *ts1* infection both in vitro and in vivo. This effect may also in part contribute to the cytoprotective effect of minocycline in infected C1 astrocytes, and to astrocyte and neuronal protection in the brain.

2.6 Minocycline prevents apoptosis in *ts1*-infected astrocytes

To determine whether minocycline prevents apoptotic signaling in the infected cells, we examined levels of apoptotic-related proteins using (a) the early markers of apoptosis

(phospho-p53 and p21), (b) levels of the anti-apoptotic protein Bcl2, and (c) the late executioner of apoptosis, which is cleaved caspase 3 (Wilson, 1998). Figure 6 shows that *ts1*-only C1 cells contained increased phospho-p53 (Ser15) and p21 at 24, 48 and 72 h of culture, while *ts1*-mino C1 cells had significantly less phospho-p53 (Ser15) and p21 at 48 and 72 h. At 72 *ts1*-only C1 cells showed pronounced cleaved caspase 3 elevations, while *ts1*-mino cells had much lower levels. Finally, although *ts1*-only C1 cells showed decreasing amounts of Bcl2 at all three timepoints, *ts1*-mino C1 cells maintained close-to-normal levels of Bcl2 throughout (Fig. 6).

2.7 Minocycline affects levels of Nrf2, COX-2 and phospho-p53 in *ts1*-infected primary astrocytes

To determine whether minocycline has similar effects on primary astrocytes to those on *ts1*-infected C1 astrocytes, we examined the expression patterns of three major proteins from minocycline-treated *ts1*-infected primary astrocytes. Similar data regarding to levels of Nrf2 were obtained from primary cultured astrocytes (Fig 7). In addition, Figure 7 also shows that similar COX-2 and phospho-p53 expression patterns were observed by Western blot from primary astrocytes with the similar treatment as C1 cells. These data indicates that effects of minocycline *ts1*-infected primary astrocytes are similar to those on *ts1*-infected C1 astrocytes.

2.8 Minocycline protects primary neurons cocultured with *ts1*-infected C1 cells, or neurons exposed to spent medium from infected C1 cell cultures

To determine whether primary neurons are affected when they are exposed to *ts1*-only C1 cells, we first set up cocultures of neurons (bottom wells) with uninfected or *ts1*-only C1 cells (top inserts). Figure 8A shows that the neurons cocultured with *ts1*-only C1 cells die. This does not occur when minocycline is added to these cocultured neurons. We next asked whether spent medium from *ts1*-only C1 cells could also harm primary neurons, and we found that it does. To find out how spent medium from infected cells kills neurons, we compared intracellular ROS levels in the minocycline treated vs. untreated neuron cultures exposed to spent medium from *ts1*-infected C1 cells. Figure 8B shows that intraneuronal ROS levels are elevated in neurons exposed to *ts1*-only C1 cell spent medium, relative to those of neuronal cultures exposed to medium from uninfected C1 cells. These results identify induced oxidative stress as one of the pathways, by which neurons are killed by nearby *ts1*-infected astrocytes. Figure 8B also shows that minocycline treatment of the neurons, given at the time when spent medium from *ts1*-only C1 cultures is added, reduces intraneuronal ROS levels, in a minocycline dose-dependent manner. Figure 8C shows that neuronal levels of Nrf2, which are reduced after addition of *ts1*-only C1 cell spent medium, are elevated by minocycline treatment. Neuronal protection by minocycline thus appears to involve antioxidant effects that include both direct radical scavenging and activation of endogenous cellular antioxidant pathways.

3. Discussion

Minocycline has been shown to reduce the incidence and severity of encephalitis in SIV-infected rhesus macaques (Zink et al., 2005), and to slow other non-viral neurodegenerative diseases (Chen et al., 2000; Du et al., 2001; Yrjanheikki et al., 1999). At present, clinical trials of minocycline in HIV-associated dementia patients are underway, however, more work is needed in nonhuman animal models for HAD regarding the mechanism of minocycline's protection in retrovirus-induced neurodegeneration. Such studies will be required to justify and strengthen the case for minocycline treatment of HIV-associated dementia, given the current controversies regarding minocycline in ND (Gordon et al., 2007; Leigh et al., 2008).

The data presented here demonstrate that minocycline drastically delays neurodegeneration in *ts1*-infected mice.

Our previous findings show that glial cells are infected by *ts1*, but neurons are not. We have proposed that the loss of neuronal function in *ts1*-induced neurodegeneration is due to (a) loss of astrocyte support, and (b) production of neurotoxic factors by infected glial cells. We have shown previously that the neurodegenerative changes in the *ts1*-infected mouse CNS are associated with the induction of oxidative stress, inflammation and apoptosis, both in vivo and in cultured astrocytes (Kim et al., 2001; Kim et al., 2005; Liu et al., 2004; Qiang et al., 2004). In this study, we asked whether neuroprotection by minocycline results from attenuation of oxidative stress, inflammation, and apoptosis in infected astrocytes. In addition, we explored the effects of *ts1*-infected astrocytes on neurons both in cocultures or after exposed them to spent medium from *ts1*-infected astrocytes. Finally, we have explored the mechanism of neuroprotection by minocycline.

We have previously reported that oxidative stress contributes to *ts1*-induced death of astrocytes, both in vivo and in vitro (Jiang et al., 2006; Qiang et al., 2004). In C1 astrocytes which survived *ts1*-infection, H₂O₂ levels are reduced, cystine uptake is increased, and higher levels of intracellular GSH and cysteine are maintained (Qiang et al., 2006). These data indicate that cells with high levels of antioxidant defenses do not die after *ts1* infection. We show here that ROS levels and lipid peroxidation are increased in *ts1*-only astrocytes. Since normal levels of redox buffer in astrocytes will provide important thiol support to nearby neurons in CNS (Aoyama et al., 2008), not surprisingly, we also show here that primary neurons underwent oxidative stress when exposed to *ts1*-infected astrocytes that failed to provide thiol support. Our data also elucidates the fact that protection of astrocytes and neurons by minocycline is associated with inhibition of oxidative stress and upregulation of thiol antioxidant defenses that are orchestrated by the transcription factor Nrf2.

Nrf2, a transcription factor that binds to antioxidant response element (ARE) sequences in the promoter regions of specific genes, regulates the transcriptional activation of genes involved in GSH synthesis, resulting in enhancement of antioxidant defenses (Qiang et al., 2004). Upregulating Nrf2 protects neurons from a variety of insults, in both cell culture and in animal models of neurodegeneration, such as PD (Jakel et al., 2007), cerebral ischemia (Shah et al., 2007; Shih et al., 2005) and ALS (Vargas et al., 2008). When minocycline is added to primary neuronal cultures immediately after adding spent *ts1*-only C1 cell medium, neuronal survival is enhanced, and intraneuronal ROS levels are reduced. Furthermore, Nrf2 levels in these neurons are upregulated by minocycline treatment. Based on these results, we suggest that decreasing levels of intraneuronal ROS and upregulation of Nrf2 are correlated with neuroprotection by minocycline. In addition, attenuation of oxidative stress by minocycline also contributes to protection of astrocytes, thus enhancing their redox support to nearby neurons.

Inflammation is another pathogenic feature in *ts1*-induced neurodegeneration (Choe et al., 1998). It is well established that while activation by various stimuli, cytoplasmic NF- κ B is disassociated from its inhibitor I κ B α and translocate to nucleus, leading to induction of expression of pro-inflammatory gene, include COX-2 (O'Neill and Kaltschmidt, 1997). We have reported that NF- κ B and COX-2 are upregulated, while I κ B α is reduced in *ts1* infected astrocytes or animals (Kim et al., 2001; Kim et al., 2005). The results reported here are consistent with these previous findings in term of alteration of these inflammatory mediators. In *ts1*-mino C1 cells, I κ B α is upregulated, thereby inhibiting nuclear translocation of NF- κ B, and suppressing production and activity of the inflammation-related molecules COX-2. Suppression of microglia activation is another mechanism associated

with the antiinflammatory effect by minocycline. This anti-inflammatory effect in *ts1*-infected astrocytes and brain may provide protection of astrocytes, reducing the possibility releasing neurotoxic factor to damage nearby neurons.

Finally, our previous data has shown that apoptosis was induced in *ts1*-infected astrocytes and animals, in association with upregulation of phospho-p53 and cleaved caspase 3 (Kim et al., 2002; Liu et al., 2004). We and others have shown that Bcl2 is downregulated in *ts1*-infected astrocytes, and that overexpression of Bcl2 protects against *ts1*-induced neurodegeneration (Jolicœur et al., 2003; Qiang et al., 2006). Our data regarding apoptosis induced by *ts1*-infection are consistent with previous findings cited above. We report here that minocycline protects *ts1*-infected C1 astrocytes by increasing their levels of Bcl2, and by reducing levels of phospho-p53, p21 and cleaved caspase 3.

In this study, we found that neuroprotection by minocycline is accompanied by reduction of viral titers in the 30 dpi infected mice brain. By contrast, although C1 astrocytic cells are protected from apoptosis by minocycline, they produce as much virus as do *ts1*-only C1 cells, while the latter are alive. We also detected similar virus titers in other organs (thymus and spleen) from *ts1*-only vs. *ts1*-mino mice. We conclude that minocycline does not directly affect virus replication, although it inhibits in vivo events associated with virus replication in these tissues. Retrovirus replication requires the phase of cellular DNA synthesis and replication in its life cycles. In the healthy CNS, potential *ts1* target cells (glial cells, including astrocytes) are quiescent, but virus-induced ROS production activates glial cells, that in turn facilitate the virus replication. Given that minocycline treatment suppresses oxidative stress in the CNS, it is likely that minocycline reduces virus replication indirectly in the CNS, by reducing ROS levels and by inhibiting activation of potential target cells (e.g. astrocytes). By contrast, our immortalized C1 cells are continuously proliferates; thus the quiescent target cell paradigm in infected mice brain is not likely to be apply for these proliferating cells, with or without minocycline treatment. These data substantiate our previous findings with another antioxidant drug, monosodium α -luminol (GVT), for which we observed that GVT treatment promotes survival in *ts1*-infected mice by its antioxidant effect (Jiang et al., 2006). Taken together, these observations support our central hypothesis that the primary causes of *ts1*-induced neurodegeneration are oxidative stress, inflammation and apoptosis resulting from virus infection, rather than from the virus load per se.

The data presented in this paper show that minocycline has a robust protective effect in CNS tissues of *ts1*-infected mice. The antioxidant, anti-inflammatory and anti-apoptotic actions of minocycline are likely to protect *ts1*-infected glial cells, particularly astrocytes. In turn, protected astrocytes could continue to supply redox support to neurons, and may reduce their production of neurotoxic excitotoxins or cytokines after infection. This mouse model for minocycline neuroprotection offers a new and unique opportunity for careful examination of minocycline as a potential HAD treatment, because the *ts1*-infected mouse is a tractable and standardized model for retrovirus-induced neurodegeneration. We have used this model, successfully identified pathways leading to neurodegeneration in *ts1*-infected mice (Jiang et al., 2006; Kim et al., 2001; Liu et al., 2004; Liu et al., 2006; Qiang et al., 2004; Qiang et al., 2006; Stoica et al., 1993). This study not only provides new evidence showing that minocycline is neuroprotective, in a mouse model of HIV-associated dementia, but it has also identified molecular targets for minocycline action.

4. Experimental procedures

4.1 Animals and infection

FVB/N mouse pups were injected intraperitoneally (i.p.) with 0.1 ml/pup *ts1* virus suspension containing 1×10^7 infectious units (IU)/ml of *ts1*, at 3 days after birth. The

infected animals were then separated into groups. *ts1*-infected minocycline-treated mice (*ts1*-mino, n=8) were treated with minocycline (Sigma), delivered intraperitoneally at 10 mg/kg/day at 5 days after birth (2 days post-infection, or dpi) continuously, until the infected untreated mice began to become paralyzed and then die. Untreated *ts1*-infected mice (*ts1*-only, n=10) were treated on the same schedule with an identical volume of normal saline. The two groups of mice were then followed for the development of paralysis and death. In mice infected at birth with this dose of *ts1*, early stages of paralysis are evident by about 30 dpi (with hindlimb drop, scored as Stage I, followed by tremor and loss of mobility at Stages II and III, and ended by the abrupt onset of Stage IV paralysis, which is brief and immediately precedes death). Mice are therefore sacrificed at Stage IV, and the time of sacrifice used as a proxy measurement for the time of survival.

4.2 Cells and minocycline treatment

Immortalized astrocytic cells of the C1 line, developed in our laboratory (Lin et al., 1997), were cultured in Dulbecco's Modified Eagle Medium (DMEM) containing 10% Fetal bovine serum (FBS). Primary astrocyte cultures were isolated from 1- to 2-day-old FVB/N mice as described previously (Shikova et al., 1993). Briefly, the cells were seeded into poly-L-lysine-coated plates and grown in DMEM-F-12 medium supplemented with 10% fetal bovine serum, 100 units/ml penicillin, 100 µg/ml streptomycin, and 2.5 µg/ml fungizone, and the medium was changed every 3 days. After reaching confluence, the cells were passed four or five times and used before reaching confluence again. At this point, more than 95% of the cells in the cultures were positive for the astrocyte-specific glial fibrillary acid protein (GFAP) marker. For infection, 10^6 cells were plated in each 100 mm plate with DMEM medium containing 1% FBS and 3 µg/ml polybrene the day before infection. The *ts1* virus was diluted in the same medium and added to the cells at a multiplicity of infection (MOI) of 10 for 40 min, followed by washing of the cells and adding DMEM medium containing 10% FBS. Minocycline was added to the medium, to a final concentration of 50 µM, immediately after *ts1* infection. Infected C1 astrocytes were tested for effects of minocycline on their survival in culture. Infected astrocytes were also cocultured with primary neurons (in dual-well chambers that prevent virus movement into the neuron-culturing medium) or were used to provide spent medium for addition to the neuron cultures. Because neurons are killed in both contexts, we tested minocycline for its ability to protect the neurons, either in cocultures or after their exposure to spent medium from the infected astrocyte cultures. 15F cells are used for virus titer assay as described previously (Jiang et al., 200a).

4.3 Virus titer determination

ts1 virus titers, in tissues and cell culture supernatants, were performed as described previously (Wong et al., 1985). Brain stems and spinal cords were taken at 30 dpi, weighed, and homogenized in 2 ml DMEM medium/sample. 15F cells were plated in DMEM medium containing 1% FBS and 3 µg/ml polybrene, and were incubated with diluted tissue filtrates for 40 min to allow attachment of virus to the 15F cells. The medium was then replaced by DMEM containing 10% FBS. The medium was changed on the 3rd day after infection. On the fifth or sixth day after infection, the foci (infectious centers) were counted and the virus titers calculated and expressed as mean $\log_{10}$ IU/g tissue $\pm$ Standard error for tissue, mean $\log_{10}$ IU/ml for cell culture supernatants.

4.4 Primary neuron cultures

Primary cortical neurons were isolated and cultured in 6-well plates as described previously (Xiang et al., 1998), with minor modifications. Briefly, cortical brain tissues from newborn FVB/N mouse pups was excised, and individual cells were dissociated initially by trypsinization (0.125% in HBSS, Ca^{2+} - and Mg^{2+} -free) for 25 min at 37°C. After washing the cells with HBSS containing Ca^{2+} and Mg^{2+} , the enzyme was inactivated with

Neurobasal medium (Invitrogen, Carlsbad, CA) containing 10% FBS. The cells were then dissociated further in serum-free Neurobasal medium plus B27 supplement (Invitrogen, Carlsbad, CA) by sequential trituration. The dissociated cells were then plated on poly-D-lysine-coated 6 well plates in serum-free Neurobasal medium plus B27 supplement, and maintained at 37°C of CO₂ incubator.

4.5 Astrocyte-neuron cocultures

C1 astrocytes were cultured on 25 mm tissue culture inserts (Nunc), whose bottoms are composed of 0.02 µm Anopore membranes. These membranes do not allow the *ts1* virus to pass from the insert chamber. For coculturing experiments, uninfected or *ts1*-infected astrocytes, cultured in Anopore inserts, were placed into receiving wells containing primary neurons cultured as described above. Both cell types were then followed over time, by microscopic evaluation, for cell death.

4.6 Western blotting analysis

C1 astrocytes, primary astrocytes or primary neurons from different treatment groups were washed with PBS and lysed in RIPA buffer as described previously (Kuang et al., 2005). Tissue lysates were cleared by centrifugation at 13,000 ×g for 20 min at 4°C. Protein concentrations were determined using the Bio-Rad Dc Protein Assay Reagent (Bio-Rad Laboratories, Hercules, CA). The lysates (30–50 µg total protein per sample) were separated on SDS-PAGE gels, transferred to PVDF membranes (Millipore Corp., Bedford, MA) and immunoblotted with primary antibodies. Nuclear and cytoplasmic extraction was performed using NE-PER Nuclear and cytoplasmic extraction kit (PIERCE) according to the manufacturer's instruction.

The primary antibodies used were anti-cleaved caspase 3, phospho-p53 (Ser15), COX-2 (all from Cell signaling), anti-malondialdehyde, or MDA (GeneTex), anti-Nrf2 (R&D), anti-Bcl2, anti-Bax, anti-p21, IκBα and NF-κB p65 (all from Santa Cruz), followed by species-specific secondary antibodies. Immune complexes were detected on the membranes using enhanced chemiluminescence (NEN Life Science Products, Boston, MA) according to the manufacturer's instructions. A monoclonal anti-β-actin antibody (Sigma) was used as a control for protein loading.

4.7 Intracellular reactive oxygen species (ROS) assay

For measurement of ROS levels in C1 astrocytes, 1.5×10^4 /C1 cells per well were plated in 96- well plates with DMEM medium, containing 1% FBS and 3 µg/ml polybrene, the day before infection. The cells were then infected at a MOI of 5 for 40 min, and either left untreated or treated with minocycline at stepped concentrations. After 4 h, the cells were washed with PBS, and the cells were loaded with 20 µM of the fluorescent probe 5-(and-6)-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate acetyl ester (CM-H₂DCFDA; Molecular Probes) for 30 min at 37 °C, followed by washing with PBS. ROS levels in the cells were measured with a fluorescent plate reader (BioTek, Winooski, Vermont) at an excitation/emission setting of 488/520 nm.

For measurement of ROS levels in neurons, primary neurons (2.5×10^5 cell/well) were plated on poly-D-lysine coated 96 well plate for 5 days. These cells were then treated with spent medium from uninfected or *ts1* -infected astrocyte cultures, in two different ratios relative to medium already in the well: 1/15 and 1/7.5. After culturing with spent medium, some cultures were left untreated, while others were treated with minocycline. 4 h later, the medium-treated neurons were washed with PBS and loaded with 20µM CM-H₂DCFDA for 20 min. Intracellular relative ROS levels were detected as described previously.

4.8 Immunohistochemistry and immunocytochemistry

Brainstem or spinal cord tissues from uninfected, *ts1*-only and *ts1*-mino animals were snap-frozen in Tissue-Tek OCT embedding medium (Electron Microscopy Sciences, Hatfield, PA) in liquid nitrogen, and cut as 5 μ m sections, stained with hematoxylin and eosin (HE) for histo-pathological analysis. For immunofluorescence staining, the sections were thawed, fixed in acetone for 5 minutes, and then incubated first in 10% donkey serum for 15 minutes, washed, and then incubated in primary antibodies rabbit anti-GFAP (sigma), or rabbit anti-CD68 (Santa Cruz), at 1:200 overnight. After incubation and washing, the sections were then incubated in anti-rabbit IgG secondary antibodies conjugated with fluorescein (anti-goat IgG; 1:200) or Texas Red from Jackson ImmunoResearch, West Grove, PA. Control sections were incubated with IgGs from rabbit serum prior to incubation in secondary antibodies, or were incubated in secondary antibodies alone. No nonspecific staining was observed on control sections (data not shown).

Immunohistochemistry with paraffin sections was performed as described previously (Kim et al., 2005). Briefly, *ts1*-only (n=5) and *ts1*-mino (n=5) mice were anesthetized at 30 dpi by intraperitoneal injection of pentobarbital (150 mg/kg), and then were transcardially perfused with 10% buffered formalin, using a peristaltic pump. After 12 h of fixation, each mouse's brain was dissected, with the brainstem segments separated for further processing. The sections (6 μ m) were deparaffinized and washed with Tris-buffered saline (TBS; 100 mM Tris, 150 mM NaCl, pH 7.4) for 20 min at room temperature. The sections were then subjected to an antigen retrieval protocol, in which they were heated in 10 mM citrate buffer (pH 6.0) for 10 min, blocked with 5% normal goat serum in TBS, and then incubated overnight at 4°C with rat anti-COX-2 antibody (Chemicon International) at a dilution of 1:100, or with rabbit anti-GFAP (Sigma; at 1:200). After three 5-min washes in TBS, the sections were then incubated with biotin-conjugated anti-rabbit or anti-goat immunoglobulin G (IgG) for 30 min at room temperature, and treated with reagents from a Vecta-Elite streptavidin peroxidase kit, with a benzidine substrate for color development. The sections were counterstained with 1% methyl green or diluted hematoxylin. Sections not incubated with a primary antibody served as negative controls.

4.9 Statistical analysis

Data are presented as means $\pm$ standard error (SEM). Cell culture experiments were conducted in triplicate or duplicate wells, with the means from three to four individual experiments used for statistical analysis. Statistical significance of the results was determined by analysis of variance (ANOVA) or Student's *t*-test. *p* values of < 0.05 were considered statistically significant. The cumulative incidence of hindlimb paralysis/death in infected mice was determined by analysis of covariance comparing slopes of curves for *ts1*-infected mice to those for infected mice treated with minocycline.

Acknowledgments

This work was supported in part by NIH Grants MH71583, NS43984 (to P.K.W.), and by NIEHS center grant ES07784, the National Cancer Institute (MD. Anderson Core Grant CA16672). We thank Christine Brown and Rebecca Deen for their assistance in preparing the manuscript. We are also most grateful to Ms. Lifang Zhang for technical assistance.

References

- Aoyama K, Watabe M, Nakaki T. Regulation of neuronal glutathione synthesis. *J Pharmacol Sci.* 2008; 108:227–328. [PubMed: 19008644]
- Chen M, Ona VO, Li M, Ferrante RJ, Fink KB, Zhu S, Bian J, Guo L, Farrell LA, Hersch SM, Hobbs W, Vonsattel JP, Cha JH, Friedlander RM. Minocycline inhibits caspase-1 and caspase-3 expression

- and delays mortality in a transgenic mouse model of Huntington disease. *Nat Med.* 2000; 6:797–801. [PubMed: 10888929]
- Choe W, Stoica G, Lynn W, Wong PK. Neurodegeneration induced by MoMuLV- ts1 and increased expression of Fas and TNF-alpha in the central nervous system. *Brain Res.* 1998; 779:1–8. [PubMed: 9473560]
- Choi Y, Kim HS, Shin KY, Kim EM, Kim M, Kim HS, Park CH, Jeong YH, Yoo J, Lee JP, Chang KA, Kim S, Suh YH. Minocycline attenuates neuronal cell death and improves cognitive impairment in Alzheimer's disease models. *Neuropsychopharmacology.* 2007; 32:2393–2404. [PubMed: 17406652]
- Clark S, Duggan J, Chakraborty J. Tsl and LP-BM5: a comparison of two murine retrovirus models for HIV. *Viral Immunol.* 2001; 14:95–109. [PubMed: 11398815]
- Dheen ST, Kaur C, Ling EA. Microglial activation and its implications in the brain diseases. *Curr Med Chem.* 2007; 14:1189–1197. [PubMed: 17504139]
- Du Y, Ma Z, Lin S, Dodel RC, Gao F, Bales KR, Triarhou LC, Chernet E, Perry KW, Nelson DL, Luecke S, Phebus LA, Bymaster FP, Paul SM. Minocycline prevents nigrostriatal dopaminergic neurodegeneration in the MPTP model of Parkinson's disease. *Proc Natl Acad Sci USA.* 2001; 98:14669–14674. [PubMed: 11724929]
- Gonzalez-Scarano, F.; Nathanson, N.; Wong, PKY. Retrovirus and the nervous system. In: Levy, J., editor. *The retrovirus.* Vol. 4. San Francisco: Plenum Press; 1995. p. 409
- Gordon PH, Moore DH, Miller RG, Florence JM, Verheijde JL, Doorish C, Hilton JF, Spitalny GM, MacArthur RB, Mitsumoto H, Neville HE, Boylan K, Mozaffar T, Belsh JM, Ravits J, Bedlack RS, Graves MC, McCluskey LF, Barohn RJ, Tandan R. Efficacy of minocycline in patients with amyotrophic lateral sclerosis: a phase III randomised trial. *Lancet Neurol.* 2007; 6:1045–1053. [PubMed: 17980667]
- Infante-Duarte C, Waiczies S, Wuerfel J, Zipp F. New developments in understanding and treating neuroinflammation. *J Mol Med.* 2008; 86:975–985. [PubMed: 18196212]
- Jakel RJ, Townsend JA, Kraft AD, Johnson JA. Nrf2-mediated protection against 6-hydroxydopamine. *Brain Res.* 2007; 1144:192–201. [PubMed: 17336276]
- Jiang Y, Scofield VL, Yan M, Qiang W, Liu N, Reid AJ, Lynn WS, Wong PK. Retrovirus-induced oxidative stress with neuroimmunodegeneration is suppressed by antioxidant treatment with a refined monosodium alpha-luminol (Galavit). *J Virol.* 2006; 80:4557–4569. [PubMed: 16611916]
- Jolicœur P, Hu C, Mak TW, Martinou JC, Kay DG. Protection against murine leukemia virus-induced spongiform myeloencephalopathy in mice overexpressing Bcl-2 but not in mice deficient for interleukin-6, inducible nitric oxide synthetase, ICE, Fas, Fas ligand, or TNF-R1 genes. *J Virol.* 2003; 77:13161–13170. [PubMed: 14645573]
- Kim HT, Qiang W, Wong PK, Stoica G. Enhanced proteolysis of IkappaBalpha and IkappaBbeta proteins in astrocytes by Moloney murine leukemia virus (MoMuLV)-ts1 infection: a potential mechanism of NF-kappaB activation. *J Neurovirol.* 2001; 7:466–475. [PubMed: 11582519]
- Kim HT, Tasca S, Qiang W, Wong PK, Stoica G. Induction of p53 accumulation by Moloney murine leukemia virus-ts1 infection in astrocytes via activation of extracellular signal-regulated kinases 1/2. *Lab Invest.* 2002; 82:693–702. [PubMed: 12065679]
- Kim HT, Qiang W, Liu N, Scofield VL, Wong PK, Stoica G. Up-regulation of astrocyte cyclooxygenase-2, CCAAT/enhancer-binding protein-homology protein, glucose-related protein 78, eukaryotic initiation factor 2 alpha, and c-Jun N-terminal kinase by a neurovirulent murine retrovirus. *J Neurovirol.* 2005; 11:166–179. [PubMed: 16036795]
- Kraus RL, Pasieczny R, Lariosa-Willingham K, Turner MS, Jiang A, Trauger JW. Antioxidant properties of minocycline: neuroprotection in an oxidative stress assay and direct radical-scavenging activity. *J Neurochem.* 2005; 94:819–827. [PubMed: 16033424]
- Kuang X, Yan M, Liu N, Scofield VL, Qiang W, Cahill J, Lynn WS, Wong PK. Control of Atm-/- thymic lymphoma cell proliferation in vitro and in vivo by dexamethasone. *Cancer Chemother Pharmacol.* 2005; 55:203–212. [PubMed: 15570424]
- Leigh PN, Meininger V, Bensimon G, Cudkowicz M, Robberecht W. Minocycline for patients with ALS. *Lancet Neurol.* 2008; 7:119–120. author reply 120–1. [PubMed: 18207106]

- Lin YC, Chow CW, Yuen PH, Wong PK. Establishment and characterization of conditionally immortalized astrocytes to study their interaction with ts1, a neuropathogenic mutant of Moloney murine leukemia virus. *J Neurovirol.* 1997; 3:28–37. [PubMed: 9147819]
- Liu N, Kuang X, Kim HT, Stoica G, Qiang W, Scofield VL, Wong PK. Possible involvement of both endoplasmic reticulum- and mitochondria-dependent pathways in MoMuLV-ts1-induced apoptosis in astrocytes. *J Neurovirol.* 2004; 10:189–198. [PubMed: 15204924]
- Liu N, Scofield VL, Qiang W, Yan M, Kuang X, Wong PK. Interaction between endoplasmic reticulum stress and caspase 8 activation in retrovirus MoMuLV-ts1-infected astrocytes. *Virology.* 2006; 348:398–405. [PubMed: 16466764]
- Mollace V, Nottet HS, Clayette P, Turco MC, Muscoli C, Salvemini D, Perno CF. Oxidative stress and neuroAIDS: triggers, modulators and novel antioxidants. *Trends Neurosci.* 2001; 24:411–416. [PubMed: 11410272]
- Morimoto N, Shimazawa M, Yamashima T, Nagai H, Hara H. Minocycline inhibits oxidative stress and decreases in vitro and in vivo ischemic neuronal damage. *Brain Res.* 2005; 1044:8–15. [PubMed: 15862784]
- Nikodemova M, Duncan ID, Watters JJ. Minocycline exerts inhibitory effects on multiple mitogen-activated protein kinases and IkappaBalpha degradation in a stimulus-specific manner in microglia. *J Neurochem.* 2006; 96:314–323. [PubMed: 16336636]
- Noble W, Garwood C, Stephenson J, Kinsey AM, Hanger DP, Anderton BH. Minocycline reduces the development of abnormal tau species in models of Alzheimer's disease. *Faseb J.* 2009; 23:739–750. [PubMed: 19001528]
- O'Neill LA, Kaltschmidt C. NF-kappa B: a crucial transcription factor for glial and neuronal cell function. *Trends Neurosci.* 1997; 20:252–258. [PubMed: 9185306]
- Qiang W, Cahill JM, Liu J, Kuang X, Liu N, Scofield VL, Voorhees JR, Reid AJ, Yan M, Lynn WS, Wong PK. Activation of transcription factor Nrf-2 and its downstream targets in response to moloney murine leukemia virus ts1-induced thiol depletion and oxidative stress in astrocytes. *J Virol.* 2004; 78:11926–11938. [PubMed: 15479833]
- Qiang W, Kuang X, Liu J, Liu N, Scofield VL, Reid AJ, Jiang Y, Stoica G, Lynn WS, Wong PK. Astrocytes survive chronic infection and cytopathic effects of the ts1 mutant of the retrovirus Moloney murine leukemia virus by upregulation of antioxidant defenses. *J Virol.* 2006; 80:3273–3284. [PubMed: 16537594]
- Reynolds A, Laurie C, Mosley RL, Gendelman HE. Oxidative stress and the pathogenesis of neurodegenerative disorders. *Int Rev Neurobiol.* 2007; 82:297–325. [PubMed: 17678968]
- Sapadin AN, Fleischnmayer R. Tetracyclines: nonantibiotic properties and their clinical implications. *J Am Acad Dermatol.* 2006; 54:258–265. [PubMed: 16443056]
- Shah ZA, Li RC, Thimmulappa RK, Kensler TW, Yamamoto M, Biswal S, Dore S. Role of reactive oxygen species in modulation of Nrf2 following ischemic reperfusion injury. *Neuroscience.* 2007; 147:53–59. [PubMed: 17507167]
- Shih AY, Li P, Murphy TH. A small-molecule-inducible Nrf2-mediated antioxidant response provides effective prophylaxis against cerebral ischemia in vivo. *J Neurosci.* 2005; 25:10321–10335. [PubMed: 16267240]
- Shikova E, Lin YC, Saha K, Brooks BR, Wong PK. Correlation of specific virus-astrocyte interactions and cytopathic effects induced by ts1, a neurovirulent mutant of Moloney murine leukemia virus. *J Virol.* 1993; 67:1137–1147. [PubMed: 8437206]
- Si Q, Cosenza M, Kim MO, Zhao ML, Brownlee M, Goldstein H, Lee S. A novel action of minocycline: inhibition of human immunodeficiency virus type 1 infection in microglia. *J Neurovirol.* 2004; 10:284–292. [PubMed: 15385251]
- Stirling DP, Khodarahmi K, Liu J, McPhail LT, McBride CB, Steeves JD, Ramer MS, Tetzlaff W. Minocycline treatment reduces delayed oligodendrocyte death, attenuates axonal dieback, and improves functional outcome after spinal cord injury. *J Neurosci.* 2004; 24:2182–2190. [PubMed: 14999069]
- Stirling DP, Koochesfahani KM, Steeves JD, Tetzlaff W. Minocycline as a neuroprotective agent. *Neuroscientist.* 2005; 11:308–322. [PubMed: 16061518]

- Stoica G, Illanes O, Tasca SI, Wong PK. Temporal central and peripheral nervous system changes induced by a paralytogenic mutant of Moloney murine leukemia virus TB. *Lab Invest.* 1993; 69:724–735. [PubMed: 8264234]
- Stoica G, Tasca SI, Wong PK. Motor neuronal loss and neurofilament-ubiquitin alteration in MoMuLV-ts1 encephalopathy. *Acta Neuropathol.* 2000; 99:238–244. [PubMed: 10663965]
- Szurek PF, Yuen PH, Ball JK, Wong PK. A Val-25-to-Ile substitution in the envelope precursor polyprotein, gPr80env, is responsible for the temperature sensitivity, inefficient processing of gPr80env, and neurovirulence of ts1, a mutant of Moloney murine leukemia virus TB. *J Virol.* 1990; 64:467–475. [PubMed: 2296075]
- Tikka T, Fiebich BL, Goldsteins G, Keinänen R, Koistinaho J. Minocycline, a tetracycline derivative, is neuroprotective against excitotoxicity by inhibiting activation and proliferation of microglia. *J Neurosci.* 2001; 21:2580–2588. [PubMed: 11306611]
- Vargas MR, Johnson DA, Sirkis DW, Messing A, Johnson JA. Nrf2 activation in astrocytes protects against neurodegeneration in mouse models of familial amyotrophic lateral sclerosis. *J Neurosci.* 2008; 28:13574–13581. [PubMed: 19074031]
- Wilson MR. Apoptosis: unmasking the executioner. *Cell Death Differ.* 1998; 5:646–652. [PubMed: 10200519]
- Wong P, Shikova E, Lin YC, Saha K, Szurek PF, Stoica G, Madden R, Brooks BR. Murine leukemia virus induced central nervous system diseases. *Leukemia.* 1992; 6(Suppl 3):161S–165S. [PubMed: 1602815]
- Wong PK, Knupp C, Yuen PH, Soong MM, Zachary JF, Tompkins WA. ts1, a Paralytogenic mutant of Moloney murine leukemia virus TB, has an enhanced ability to replicate in the central nervous system and primary nerve cell culture. *J Virol.* 1985; 55:760–767. [PubMed: 4020966]
- Wong PK, Floyd E, Szurek PF. High susceptibility of FVB/N mice to the paralytic disease induced by ts1, a mutant of Moloney murine leukemia virus TB. *Virology.* 1991; 180:365–371. [PubMed: 1984656]
- Wong PK, Yuen PH. Cell types in the central nervous system infected by murine retroviruses: implications for the mechanisms of neurodegeneration. *Histol Histopathol.* 1994; 9:845–858. [PubMed: 7894152]
- Wong, PKY. *Current Topics in Microbiology and Immunology.* Vol. Vol. 160. Heidelberg: Springer-Verlag Berlin; 1990. Moloney Murine Leukemia Virus temperature-sensitive mutants, a model for retrovirus-induced neurologic disorders; p. 29
- Wong, PKYYP. *Molecular Basis of Neurologic Disorders induced by a utant, ts1, of Moloney Murine Leukemia Virus.* In: RP, R., editor. *Molecular Neurovirology: Pathogenesis of viral CNS infections.* Vol. Vol. Chicago: Humana Press; 1992. p. 161
- Xiang H, Kinoshita Y, Knudson CM, Korsmeyer SJ, Schwartzkroin PA, Morrison RS. Bax involvement in p53-mediated neuronal cell death. *J Neurosci.* 1998; 18:1363–1373. [PubMed: 9454845]
- Yong VW, Wells J, Giuliani F, Casha S, Power C, Metz LM. The promise of minocycline in neurology. *Lancet Neurol.* 2004; 3:744–751. [PubMed: 15556807]
- Yrjanheikki J, Tikka T, Keinänen R, Goldsteins G, Chan PH, Koistinaho J. A tetracycline derivative, minocycline, reduces inflammation and protects against focal cerebral ischemia with a wide therapeutic window. *Proc Natl Acad Sci U S A.* 1999; 96:13496–13500. [PubMed: 10557349]
- Zachary JF, Baszler TV, French RA, Kelley KW. Mouse Moloney leukemia virus infects microglia but not neurons even though it induces motor neuron disease. *Mol Psychiatry.* 1997; 2:104–106. [PubMed: 9106227]
- Zhu S, Stavrovskaya IG, Drozda M, Kim BY, Ona V, Li M, Sarang S, Liu AS, Hartley DM, Wu DC, Gullans S, Ferrante RJ, Przedborski S, Kristal BS, Friedlander RM. Minocycline inhibits cytochrome c release and delays progression of amyotrophic lateral sclerosis in mice. *Nature.* 2002; 417:74–78. [PubMed: 11986668]
- Zink MC, Uhrlaub J, DeWitt J, Voelker T, Bullock B, Mankowski J, Tarwater P, Clements J, Barber S. Neuroprotective and anti-human immunodeficiency virus activity of minocycline. *Jama.* 2005; 293:2003–2011. [PubMed: 15855434]

Fig. 1.

Minocycline delays paralysis and spongiform lesion formation in *ts1*-infected mice. (A) The survivor curves shows *ts1-mino* group (*ts1-mino*, n=8) against the same survivor curve for *ts1-only* mice (*ts1*, n=10). FVB/N mouse pups were injected *ts1* virus i.p. at 3 days after birth. 8 infected mice were treated with minocycline 10mg/kg/day i.p. at 5 days after birth continuously until mice die. *** $p < 0.001$ for *ts1-only* mice vs. *ts1-mino* mice. (B) Frozen sections of brain stem and spinal cord from different groups of mice were stained with hematoxylin and eosin (H&E) for histopathological examination. Spongiform lesions were apparent in *ts1-only* mice brainstem and spinal cord, while the *ts1-mino* mice showed a few small vacuoles, but no discrete lesions. (C) Frozen sections of brainstem from different

groups of mice were stained with GFAP antibody. Astrogliosis are apparent in *ts1*-only section, but attenuated in *ts1*-mino section.

Fig. 2. Minocycline protects *ts1*-infected astrocytes, (a). Viability assay by trypan Blue indicated that minocycline protect *ts1*-infected astrocytes. C1 astrocytes were infected with *ts1* at a MOI of 10, minocycline was added at a concentration of 50 μ M. Viability assays were performed at 24, 48 and 72 h post infection. * $p < 0.05$; *** $p < 0.001$ when compared with uninfected cells. ## $p < 0.01$ when compared with *ts1*-only cells.

Fig. 3.

Minocycline decreases virus titers in the CNS, but not in thymus and spleen from mice, and infected C1 cells. (A). FVB/N mouse pups were infected with *ts1*, then either left untreated or treated with minocycline as described above. Brain stems (BS), spinal cords (SC), spleen and thymi were harvest and subjected to virus titer assays. The results shown are the means $\pm$ SEM from four to seven mice for each group. *** $p < 0.001$. (B). C1 astrocytes were infected with *ts1* at a MOI of 10, minocycline was added at a concentration of 50 μ M. Supernatants were collected at 24, 48 and 72h, and subjected to virus titer assays.

Fig. 4.

Minocycline has antioxidant effects on *ts1*-infected astrocytes. (A). Minocycline decreased the elevated ROS levels in H₂O₂-treated (100 μM for 1 hr) astrocytes. Relative ROS levels were detected by loading with CM-H₂DCFDA, followed by measurement of the relative intensity of fluorescent signals. #*p*<0.05 when compared with uninfected cells. **p*<0.05; ***p*<0.01 when compared with H₂O₂-treated cells. (B). Lower ROS levels are present in *ts1*-mino C1 cells. #*p*<0.05 when compared with uninfected cells. **p*<0.05 when compared with *ts1*-only C1 cells. (C). MDA levels were reduced in *ts1*-mino cells. Western blotting was used to detect MDA levels. (D). Western blotting analysis show that Nrf2 levels are decreased in *ts1*-infected astrocytes, while minocycline upregulated levels of Nrf2.

Fig. 5.

Minocycline has anti-inflammatory effects in *ts1*-infected mice brainstem and cultured C1 astrocytes. (A). Cell lysates or nuclear extracts from uninfected, *ts1*-only and *ts1*-mino C1 astrocytes are probed with anti-I κ B α , NF- κ B p65 and COX-2. (B). Mice from two groups were sacrificed at 30 dpi. Paraffin sections of the brainstem used for immunohistochemistry (IHC) assay. These sections were incubated with anti-GFAP (brown) and anti-COX-2 antibodies as specified in Experimental Procedures. Strong COX-2 immunoreactivity (red) is evident in astrocyte (arrow) of *ts1*-only mouse (left panel), but weak in *ts1*-mino mouse (right panel). Magnifications: 40X. (C). Expression of CD68, which marks activated microglia and macrophages, was examined by immunofluorescence staining using brainstem sections from uninfected, *ts1*-only and *ts1*-mino mice. Magnifications: 20X.

Fig. 6. Minocycline prevents apoptosis in *ts1* -infected C1 astrocytes. Apoptotic pathway related proteins phospho-p53, p21, Bcl-2, Bax and cleaved caspase3 were analyzed by Western blotting.

Fig. 7.

Levels of Nrf2, COX-2 and phospho-p53 were analyzed by Western blot using lysates from uninfected, *ts1*-infected and infected minocycline-treated primary astrocytes. Primary astrocytes were infected with *ts1* virus at an MOI of 10 and treated with minocycline at 5 μ M for 96 h and cell lysates were subjected to Western blot analysis.

Fig. 8.

Minocycline treatment protects primary neurons. (A) Representative photomicrographs of cultured primary neurons show that minocycline (25 μ M) protects neurons from cell death when they are cocultured with *ts1*-infected C1 astrocytes. (B) Minocycline treatment maintains normal ROS levels in primary neurons exposed to spent *ts1*-infected astrocyte medium. # $p < 0.05$ and ## $p < 0.01$ when compared with uninfected cells. * $p < 0.05$; ** $p < 0.01$ when compared with 1/7.5 *ts1*-C1 spent medium-treated cells. (C) Nrf2 levels were upregulated in minocycline treated primary neurons after exposed to spent medium from *ts1*-infected astrocytes. Western blotting is used for detecting levels of Nrf2.