A RAT MODEL OF STAVUDINE-INDUCED HYPERALGESIA

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DECLARATION

This thesis is submitted in the optional format, approved by the Faculty of Science, of published and submitted work with supporting introduction and conclusion.

I declare that this thesis is my own unaided work, unless otherwise specified. It is being submitted for the Degree of Doctor of Philosophy at the University of the Witwatersrand, Johannesburg.

This work has not been submitted before for any degree or examination in any other university.

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ABSTRACT

Stavudine, a nucleoside reverse transcriptase inhibitor (NRTI) used to treat infection by the human immunodeficiency virus (HIV), causes peripheral neuropathy and pain in HIV-positive patients. The mechanisms of this toxic neuropathy are poorly understood, partly because of a lack of animal models of the disease process. I investigated whether long-term daily oral administration of stavudine affects nociception in Sprague-Dawley rats, and whether changes in nociception are accompanied by a general deterioration in the rats' conditions, as reflected in activity and appetite. Daily stavudine administration induced mechanical hyperalgesia in rats within three weeks without affecting appetite, growth or physical activity, and this hyperalgesia persisted throughout the six weeks of stavudine administration. I then investigated whether central changes underlie the hyperalgesia caused by stavudine in rats by examining inflammatory cytokine secretion and neuronal death in the spinal cord. Daily stavudine administration caused an increase in cytokine-induced neutrophil chemo-attractant (CINC)-1 concentration in the spinal cord after six weeks, but early development of stavudine-induced hyperalgesia did not depend on increases in spinal concentrations of CINC-1 and interleukin (IL)-6, nor on apoptosis or necrosis of spinal neurones. The neurotoxicity of stavudine is thought to derive from mitochondrial toxicity, which has been linked to increased plasma lactate concentration and decreased plasma adiponectin levels caused by lipodystrophy. Thus, I investigated whether a systemic inflammatory response or metabolic dysregulation accompanied

stavudine-induced hypernociception by examining plasma adiponectin, lactate, CINC-1 and IL-6 concentrations in rats administered daily stavudine. Plasma adiponectin, lactate, CINC-1 and IL-6 concentrations were unchanged following three or six weeks of daily stavudine administration. Therefore, I have shown that stavudine-induced hyperalgesia is not dependent on spinal cord plasticity, nor on a systemic inflammatory response or extensive metabolic malfunction. Instead, the hyperalgesia I observed may be caused by the adverse effects of stavudine on peripheral neurone functioning. As stavudine administration to healthy rats had no adverse effects besides inducing hyperalgesia and causing a rise in CINC-1 concentration in the spinal cord after six weeks, my results indicate that many other side effects commonly associated with stavudine treatment in HIV-positive patients may arise through interaction with the underlying HIV infection.

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LIST OF ABBREVIATIONS

AIDS	acquired immunodeficiency syndrome
ANCOVA	Analysis of Covariance
ANOVA	Analysis of Variance
CINC	cytokine-induced neutrophil chemo-attractant
DAB	diaminobenzidine
ELISA	enzyme-linked immunosorbent assay
gp120	glycoprotein 120
HAART	highly active antiretroviral therapy
H&E	haematoxylin and eosin
HIV	human immunodeficiency virus
HRP	horseradish peroxidase-labeled
IDV	indinavir
IL	interleukin
i.p.	intraperitoneal
JNK	c-Jun N-terminal kinase
NNRTI	non-nucleoside reverse transcriptase inhibitor
NRTI	nucleoside reverse transcriptase inhibitor
PBS	phosphate buffered saline
PI	protease inhibitor

RANTES	regulated upon activation, normal T-cell expressed and secreted
rTDT	terminal Deoxynucleotidyl Transferase
SSC	standard saline citrate
TNF	tumour necrosis factor
TUNEL	Terminal Deoxynucleotidyl Transferase-Mediated dUTP Nick End-Labelling
WHO	World Health Organisation

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RESEARCH OUTPUTS

Journal publications

Weber J., Mitchell D. and Kamerman P.R. (2007). Oral administration of stavudine induces hyperalgesia without affecting activity in rats. *Physiology and Behavior*, 92:807–813.

Published abstracts

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Submitted papers

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Weber J., Mitchell D. and Kamerman P.R. Oral administration of the antiretroviral drug stavudine results in mechanical hyperalgesia and increased CINC-1 in the spinal cord of rats. 34th meeting of the Physiology Society of Southern Africa (PSSA), 26-29 September 2006, University of KwaZulu-Natal, Durban, South Africa (poster presentation).

Weber J., Mitchell D., Mitchell B. and Kamerman P.R. Oral administration of stavudine induces mechanical hyperalgesia and increased CINC-1 in the spinal cord of rats. 2nd International Congress on Neuropathic Pain (NeuPSIG), 7-10 June 2007, Berlin, Germany (poster presentation).

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Weber J., Mitchell D., Veliotes D., Mitchell B. and Kamerman P.R. Hyperalgesia induced by oral administration of stavudine to rats does not depend on release of spinal pro-inflammatory cytokines, nor on spinal neuronal apoptosis or necrosis. 35th meeting of the Physiology Society of Southern Africa (PSSA), 9-12 September 2007, Glenburn Lodge, Muldersdrift, South Africa (poster presentation).

AUTHORS' CONTRIBUTIONS

The contributions of each author to the published or submitted papers contained in this thesis are listed below.

Chapter two

Paper one: Weber J., Mitchell D. and Kamerman P.R. (2007). Oral administration of stavudine induces hyperalgesia without affecting activity in rats. *Physiology and Behavior*, 92:807–813.

Experimental design and preparation, all data collection and analysis, and drafting of the manuscript were performed by me, with guidance from my supervisors, D Mitchell and PR Kamerman.

Chapter three

Paper two: Weber J., Mitchell D., Veliotes D., Mitchell B. and Kamerman P.R. Hyperalgesia induced by oral stavudine administration to rats does not depend on spinal release of the pro-inflammatory cytokines interleukin-6 or cytokine-induced neutrophil chemo-attractant-1, nor on spinal neuronal apoptosis or necrosis. Submitted to *Antiviral Therapy* on 27 February 2008.

Experimental design and preparation, behavioural data collection, tissue sample collection, staining of spinal cord sections, determination of TUNEL-positive nuclei and cytokine concentrations, data analysis and drafting of the manuscript were performed by me, with guidance from my supervisors, D Mitchell and PR

Kamerman. D Veliotes assisted with the TUNEL staining and interpretation of these results. B Mitchell, a pathologist, examined spinal cord sections for signs of damage and counted neuronal nuclei.

Chapter four

Paper three: Weber J., Mitchell D. and Kamerman P.R. Oral administration of stavudine to rats at a dose inducing hyperalgesia does not affect plasma lactate, adiponectin, cytokine-induced neutrophil chemo-attractant-1 or interleukin-6 concentrations. Submitted to *Antiviral Therapy* on 27 February 2008.

Experimental design and preparation, collection of tissue samples, lactate, adiponectin and cytokine assays, data analysis and drafting of the manuscript were performed by me, with guidance from my supervisors, D Mitchell and PR Kamerman.

CHAPTER ONE

INTRODUCTION

Pain is a common complaint of HIV-positive patients, even in the absence of AIDS-defining diseases, and frequently is underestimated and treated poorly by doctors (Breitbart et al., 1996; Del Borgo et al., 2001; Husstedt et al., 2001; Karus et al., 2005; Larue et al., 1997). The prevalence of pain in HIV-positive patients increases from about 30 % in adult out-patients, to about 60 % in adult in-patients (Brechtl et al., 2001; Breitbart et al., 1996; Karus et al., 2005; Larue et al., 1997). Norval (2004) found that in South Africa 98 % of patients with advanced AIDS in a palliative care facility suffered from chronic pain. Despite the high prevalence of pain in HIV-positive patients, 85 % of those patients are not given adequate pain relief by doctors (Breitbart et al., 1996; Larue et al., 1997).

While somatic pain, headache, joint pain and muscle pain frequently are reported by HIV-positive patients, approximately 30 % of HIV-related pain is thought to be neuropathic in origin (Del Borgo et al., 2001; Hewitt et al., 1997), not only because of neural damage caused by the virus, but also because some antiretroviral drugs cause toxic neuropathies (Cherry et al., 2003; Dalakas, 2001). Although antiretroviral drugs effectively retard the progression of the disease, some studies have shown that the prevalence of sensory neuropathy in HIV-positive patients has increased since the introduction of these drugs (Bacellar et al., 1994; Husstedt et al., 2001; Luciano et al., 2003; Sacktor et al., 2001; Smyth et al., 2007). Clinically, distal sensory polyneuropathy caused by the virus and toxic neuropathy caused by antiretroviral drugs are indistinguishable (Dalakas, 2001; McArthur et al., 2005;

Nicholas et al., 2007; Simpson & Tagliati, 1995), as both types of neuropathy are associated with the same features, including decreased peripheral nerve fibre density (Cherry et al., 2003; Pardo et al., 2001; Polydefkis et al., 2002; Zhou et al., 2007). A diagnosis of toxic neuropathy is dependent mainly on when the symptoms of neuropathic pain occur, in relation to the start of antiretroviral therapy (McArthur et al., 2005; Nicholas et al., 2007).

While highly active antiretroviral therapy (HAART) used to treat HIV infection usually consists of a combination of two nucleoside reverse transcriptase inhibitors (NRTIs) and either a non-nucleoside reverse transcriptase inhibitor (NNRTI) or a protease inhibitor (PI) (Grimwood, 2004; WHO, 2004), NRTIs are associated with a greater risk of developing neuropathy than any other antiretroviral drug (Bacellar et al., 1994; Keswani et al., 2002; Moore et al., 2000). Of the other classes of antiretroviral drugs only the PI indinavir (IDV) recently has been found to be a risk factor for developing peripheral neuropathy (Smyth et al., 2007). Although NRTIs form an integral part of HAART, the mechanisms of NRTI-induced toxic neuropathy are not well understood. Our poor understanding of how the toxicity of NRTIs causes pain in HIV-positive patients arises partly because of a lack of robust animal models of the disease process. The purpose of this introduction is to describe the clinical features of distal sensory polyneuropathy caused by the HI virus and by antiretroviral drugs. As neuropathic pain caused by antiretroviral drugs is the focus of my PhD, in this chapter I will examine the role of NRTI-related mitochondrial

toxicity on NRTI-induced toxic neuropathy and illustrate the effects of NRTIs on nerve fibre density in animals and nerve fibre growth in cell culture. As it is impossible to distinguish between the effects of the virus and the antiretroviral drugs on neurones in HIV-positive patients, I also will describe animal models of NRTI-induced pain hypersensitivity, which have been developed to characterise toxic neuropathy in the absence of HIV infection.

1.1 Peripheral neuropathy in HIV-positive patients

Somatic pain, headache, joint pain, muscle pain and neuropathic pain are among the most frequently reported types of pain experienced by HIV-positive patients, with about 30 % of patients suffering from neuropathic pain (Del Borgo et al., 2001; Hewitt et al., 1997). Peripheral neuropathy, with or without pain, affects up to 50 % of HIV-positive adult patients (Dagan et al., 2002; Simpson et al., 2003; Smyth et al., 2007), with the incidence of neuropathy increasing with disease progression and decreased CD4 cell count (Breitbart et al., 1996; Hewitt et al., 1997; Larue et al., 1997). The occurrence of peripheral neuropathy also is increased in HIV-positive patients with a history of other types of neuropathy (Stenzel & Carpenter, 2000) and with increased age (Smyth et al., 2007). The prevalence of peripheral neuropathy is similar in HIV-positive adults and children, although the condition frequently is less severe in children than in adults (Araujo et al., 2000).

One of the signs of peripheral neuropathy is a reduction in epidermal nerve fibre

density, caused by distal axonal degeneration (Cherry et al., 2003; Pardo et al., 2001; Polydefkis et al., 2002; Zhou et al., 2007). Nerve fibre degeneration is accompanied by the release of local inflammatory mediators, which heighten the sensitivity of surrounding, uninjured fibres, contributing to neuropathic pain (Keswani et al., 2002). Initially, small unmyelinated nerve fibres are lost, followed by the degeneration of large myelinated fibres (Cherry et al., 2003). Regeneration of damaged nerve fibres is rare (McArthur et al., 2005) and regrowth of damaged nerve fibres appears to be impaired with HIV infection (Hahn et al., 2007). Hahn and colleagues (2007) found that cutaneous capsaicin application resulted in near complete denervation of the affected area and that regeneration of these nerve fibres was significantly decreased in HIV-positive patients with or without neuropathy compared to healthy controls.

Approximately 50 % of HIV-positive patients with peripheral neuropathy suffer from pain (Cornblath & McArthur, 1988; Fuller et al., 1993; Martin et al., 2003), most prominently in the lower limbs (Norval, 2004). Patients experience hyperalgesia and allodynia, impaired thresholds for hot and cold stimulation, and numbness and burning of the extremities, particularly the feet (Cherry et al., 2005; Huengsberg et al., 1998; Martin et al., 2003; McArthur et al., 2005). HIV-positive patients frequently experience spontaneous pain (Martin et al., 2003; Schreiner & McCormick, 2002), making it difficult to perform normal daily activities, diminishing quality of life and often resulting in anxiety and depression (Larue et al., 1997; Newshan et al., 2002; Ownby & Dune, 2007).

While most HIV-positive patients who have died of AIDS have detectable nerve damage, indicative of peripheral neuropathy, not all of these patients reported experiencing pain when they were alive (Keswani et al., 2002; Luciano et al., 2003; Martin et al., 2003). Non-painful peripheral neuropathy may be the result of partial damage to the nervous system and limited C-fibre dysfunction, allowing for relatively normal nociception (Martin et al., 2003). Alternatively, asymptomatic neuropathy may be caused by extensive neural damage, which decreases sensory input, such that the patient is less sensitive to stimuli and does not suffer from pain (Pardo et al., 2001).

1.2 gp120-induced neurotoxicity

Much of what is known about the mechanisms of HIV-induced neuropathy and the pain associated with that neuropathy was discovered using animal models. The HIV envelope glycoprotein gp120, injected intrathecally or administered perineurally to mice and rats, results in mechanical and thermal hyperalgesia (Herzberg & Sagen, 2001; Milligan et al., 2000; Milligan et al., 2001a; Milligan et al., 2001b; Spataro et al., 2004; Twining et al., 2005), mechanical allodynia (Herzberg & Sagen, 2001; Ledeboer et al., 2005; Minami et al., 2003; Wallace et al., 2007a), and a decrease in intra-epidermal nerve fibre density (Wallace et al., 2007b). HIV-gp120 induces macrophage infiltration in the peripheral nerve and dorsal root ganglia (Wallace et al., 2007a) and activates microglia and astrocytes in the brain and spinal cord (Herzberg & Sagen, 2001; Ledeboer et al., 2005; Milligan et al., 2000; Milligan et al., 2001b; Wallace et al., 2007a), which then release the pro-inflammatory

cytokines interleukin (IL)-1, tumour necrosis factor (TNF)-α (Herzberg & Sagen, 2001; Holguin et al., 2004; Milligan et al., 2001a; Milligan et al., 2001b) and IL-6 (Holguin et al., 2004), as well as the chemokine CCL2 (Wallace et al., 2007b). The release of nitric oxide (Holguin et al., 2004) and calcium (Minami et al., 2003) in the spinal cord also is increased following gp120 administration and in addition the spinal cord complement cascade is activated (Twining et al., 2005). Inhibiting the complement cascade and glial cell activity and blocking the release of nitric oxide and cytokines reduces or abolishes the heightened pain sensitivity induced by gp120 (Holguin et al., 2004; Ledeboer et al., 2005; Milligan et al., 2000; Milligan et al., 2001b; Minami et al., 2003; Twining et al., 2005), highlighting the importance of centrally-mediated pathways in HIV-related neuropathic pain.

HIV-gp120 also may directly affect neurone functioning. Isolated rat dorsal root ganglion neurones incubated with gp120 show an increase in neuronal apoptosis (Bodner et al., 2004; Keswani et al., 2003b), a decrease in neurite branching and total neurite length (Keswani et al., 2003b) and an increase in the release of substance P and calcium (Oh et al., 2001), which may contribute to the increased pain sensitivity observed in rodents administered gp120. Chemokines are thought to play an essential role in HIV-induced neuropathy, as blocking chemokine receptors reduces the adverse effects of gp120 on neurone viability (Bodner et al., 2004; Keswani et al., 2003b). HIV-gp120 is thought to bind to the CXCR4 chemokine receptor on Schwann cells, resulting in the release of the chemokine RANTES (regulated upon activation, normal T-cell expressed and secreted), which binds to

the CCR5 chemokine receptor on neurones, resulting in the release of TNF-α and subsequent neurotoxic effects (Keswani et al., 2003b).

These animal and cell culture studies of gp120-induced neurotoxicity show that the HIV envelope glycoprotein gp120 plays an important role in mediating the peripheral neuropathy caused by HIV infection. By causing the release of neurotoxic substances, such as pro-inflammatory cytokines and chemokines, gp120 enhances the sensitivity of neurones and may result in neuronal death, features evident in HIV-positive patients with painful peripheral neuropathy caused by the virus. Because of a dearth of similar animal models of antiretroviral drug-induced neuropathy, the mechanisms of toxic neuropathy are not as well understood.

1.3 Toxic neuropathy caused by antiretroviral drugs

Although antiretroviral drugs effectively delay the development of AIDS and increase life expectancy by increasing CD4 cell count and decreasing viral load (Brechtl et al., 2001; Dalakas, 2001), other symptoms of HIV-infection, such as fatigue, insomnia, anxiety, and pain, have not been eliminated (Brechtl et al., 2001; Newshan et al., 2002). Indeed, some studies have shown that the incidence of peripheral neuropathy has increased with the introduction of antiretroviral drugs (Bacellar et al., 1994; Husstedt et al., 2001; Sacktor, 2002; Sacktor et al., 2001; Smyth et al., 2007). Researchers believe that, in HIV-positive patients, the toxic effects of antiretroviral drugs may merely enhance a previously existing distal sensory polyneuropathy caused by the virus (Keswani et al., 2002), such that HIV

infection is necessary for toxic neuropathy to develop. The virus, pro-inflammatory cytokines and chemokines are thought to cause the initial peripheral nerve injury (see section 1.2), which then is exacerbated by the toxic effects of antiretroviral drugs, resulting in symptomatic, painful neuropathy (Keswani et al., 2002).

While HAART usually consists of a combination of two NRTIs, the backbone of combination therapy (Sension, 2007), and an NNRTI or PI (Grimwood, 2004; WHO, 2004), the toxic effects of NRTIs in particular are associated with the high incidence of peripheral neuropathy in HIV-positive patients taking antiretroviral drugs (Bacellar et al., 1994; Keswani et al., 2002; Moore et al., 2000; Smyth et al., 2007). Toxic neuropathy often arises rapidly (Dalakas, 2001) six to eight weeks after starting NRTI treatment (Husstedt et al., 2001), although neuropathy may develop as early as one week or up to six months after the onset of NRTI administration (Keswani et al., 2002). This temporal association between the symptomatic development of neuropathy and the start of antiretroviral therapy currently is necessary for the diagnosis of NRTI-induced neuropathy, as the clinical symptoms of toxic neuropathy and distal sensory polyneuropathy, caused by the virus, are identical (Dalakas, 2001; McArthur et al., 2005; Nicholas et al., 2007; Simpson & Tagliati, 1995). Kokotis and colleagues (2007) recently showed that, although the clinical symptoms of both kinds of neuropathy are the same, the pathology of toxic neuropathy and distal sensory polyneuropathy may differ. The researchers found that neuropathy caused by the virus was associated largely with a decrease in the conduction velocity of myelinated nerve fibres, while toxic

neuropathy appeared to decrease the conduction velocity mainly of unmyelinated fibres. Although the authors failed to demonstrate a clear distinction between the two types of neuropathy (Kokotis et al., 2007), similar studies may allow us to discriminate between neuropathy caused by HIV infection and that resulting from antiretroviral drug treatment in the future, which may improve the management of the condition.

The prevalence of toxic neuropathy increases with the number of antiretroviral drugs used (Scarsella et al., 2002), the duration of NRTI exposure (Scarsella et al., 2002) and frequent changing of HAART regimens (Silverberg et al., 2004). Also, HIV-positive patients with advanced HIV disease, increased age, decreased nutritional status (Fichtenbaum et al., 1995; Moyle & Sadler, 1998) and any previous incidence of neuropathy are at greater risk of developing toxic neuropathy (Fichtenbaum et al., 1995; Keswani et al., 2002). Discontinuing the use of NRTIs, or changing the HAART regimen (Keswani et al., 2002), results in an improvement of symptoms in two-thirds of patients (Keswani et al., 2002), usually within three months (Blum et al., 1996).

Table 1: Commonly used* nucleoside reverse transcriptase inhibitors (NRTIs)

Generic names	**Chemical name**	**Nucleotide derivative**	**Brand name**	**Manufacturer**
Didanosine (ddI)	2',3'-dideoxyinosine	Adenosine	Videx EC	Bristol-Myers Squibb
Lamivudine (3TC)	B-L-2',3'-dideoxy-3'-thiacytidine	Cytidine	Epivir	IAF Biochem Int/ Glaxo Wellcome
Stavudine (d4T)	2',3'-didehydro-3'-deoxythymidine	Thymidine	Zerit	Bristol-Myers Squibb
Zalcitabine (ddC)*	2',3'-dideoxycytidine	Cytidine	Hivid	Hoffman La Roche
Zidovudine (AZT)	3'-azido-3'-deoxythymidine	Thymidine	Retrovir	Glaxo Wellcome

*zalcitabine no longer is recommended for the treatment of HIV infection

All NRTIs (see Table 1 for brand names and manufacturers), except zidovudine (AZT), which is administered at a dose of 600 mg per day (WHO, 2004), have been associated with peripheral neuropathy (Dalakas, 2001). Besides causing toxic neuropathy NRTIs also are associated with numerous other adverse effects, including gastrointestinal disturbances, pancreatitis, lactic acidosis, lipodystrophy and rash (Montessori et al., 2004), however neuropathy is one of the most common reasons for discontinuing the use of these drugs. The prevalence of peripheral neuropathy and other side effects commonly reported with the use of NRTIs is summarised in Table 2. The severity and the prevalence of the peripheral neuropathy caused by NRTIs is dependent on the toxicity of the specific NRTI used (Dalakas, 2001). Zalcitabine (ddC) is associated with the highest incidence of neuropathy, with all patients administered a high dose ($\geq$ 0.12 $mg.kg^{-1}$ per day) developing neuropathy in seven to nine weeks. Even with a much lower dose (0.02 $mg.kg^{-1}$ per day) neuropathy developed in one third of patients within 26 weeks (Berger et al., 1993). The neuropathy caused by zalcitabine is painful, progresses rapidly and may be irreversible (Cherry et al., 2003; Dalakas, 2001). Because of the high incidence of toxic neuropathy associated with zalcitabine administration, zalcitabine no longer is recommended for the treatment of HIV infection. Neuropathy occurs less frequently with the use of lamivudine (3TC), didanosine (ddI) and stavudine (d4T) and the neuropathy generally improves when treatment is stopped (Cherry et al., 2003; Dalakas, 2001). The prevalence of neuropathy caused by lamivudine, which is administered at a dose of 300 mg per day (WHO, 2004), has not been assessed systematically (Dalakas, 2001). The recommended dose of

didanosine is 250 or 400 mg per day, depending on the patient's body mass (WHO, 2004). A dose of didanosine far greater than that currently recommended (≥ 12 $mg.kg^{-1}$ per day) resulted in neuropathy in up to 50 % of patients (Lambert et al., 1990), but neuropathy affected only 2 % of patients administered didanosine at recommended doses (Moyle & Sadler, 1998) and those with higher CD4 cell counts administered a much lower dose (0.4 $mg.kg^{-1}$ per day) of this NRTI (Lambert et al., 1990). A high dose (approximately four times the currently recommended dose) of stavudine caused peripheral neuropathy in 70 % of HIV-positive patients (Skowron, 1995), while currently recommended therapeutic doses of 60 or 80 $mg.day^{-1}$ (WHO, 2004) may cause neuropathy in up to 15 % of patients (Moyle & Sadler, 1998; Simpson & Tagliati, 1995).

Table 2: Prevalence of peripheral neuropathy and other common side effects caused by NRTI administration in HIV-positive patients

NRTI	Dose	Patients with peripheral neuropathy (%)	Other common side effects[6]
Didanosine	≥ 12 mg.kg^{-1} per day[1]	50	Gastrointestinal disturbances, pancreatitis, hepatic steatosis, lactic acidosis
	0.4 mg.kg^{-1} per day[1]; 250 or 400 mg per day[2]	2	
Lamivudine	300 mg per day	Not assessed	Neutropenia
Stavudine	Four times currently recommended[3]	70	Gastrointestinal disturbances, lactic acidosis, lipodystrophy, hepatic steatosis
	60 or 80 mg per day[2,4]	15	
Zalcitabine	≥ 0.12 mg.kg^{-1} per day[5]	100	Pancreatitis, mouth ulcers, lactic acidosis
	0.02 mg.kg^{-1} per day[5]	33	
Zidovudine	600 mg per day	None reported	Gastrointestinal disturbances, rash, anaemia, lactic acidosis, hepatotoxicity

References: 1. Lambert et al., 1990; 2. Moyle & Sadler, 1998; 3. Skowron, 1995; 4. Simpson & Tagliati, 1995; 5. Berger et al., 1993; 6. Montessori et al., 2004

1.4 Mechanism of action of NRTIs

NRTIs are derivatives of the nucleotides adenosine, cytidine, guanosine or thymidine (see Table 1), with a modified 3',OH end of the deoxyribose sugar (Kakuda, 2000). NRTIs need to be phosphorylated before they can exert their effects, and this process is specific to each NRTI, resulting in different rate-limiting steps in the actions of different NRTIs (Kakuda, 2000). The active, triphosphorylated form of an NRTI provides an alternative substrate for DNA polymerases, including the HIV reverse transcriptase enzyme (Dagan et al., 2002). By competing with normal nucleic acids and terminating chain elongation before completion, NRTIs prevent the virus from producing DNA copies of its RNA, impairing viral replication (Dagan et al., 2002; Kakuda, 2000).

Although NRTIs successfully decrease viral proliferation, the inhibitory effect of these antiretroviral drugs is not restricted to viral enzymes. Just as NRTIs decrease viral DNA production by inhibiting the HIV reverse transcriptase enzyme, NRTIs also may decrease mitochondrial DNA production by inhibiting mitochondrial enzymes, resulting in mitochondrial dysfunction.

1.5 Effects of NRTIs on mitochondrial function

The metabolic abnormalities and decreased ATP production caused by NRTI-induced mitochondrial dysfunction may have debilitating effects, leading to tissue and organ malfunction (Cherry et al., 2003; McComsey & Lonergan, 2004), including nerve damage causing peripheral neuropathy and resulting in pain

(Cherry et al., 2003). NRTI-mediated mitochondrial dysfunction is linked to lactic acidosis (Montessori et al., 2004), a common side effect of NRTI use (see Table 2), which is caused by an increase in anaerobic respiration and lactate buildup (Dagan et al., 2002; Lewis, 2003; McComsey & Lonergan, 2004). Lipodystrophy, the fat redistribution characterised by peripheral fat loss and central fat accumulation, in HIV-positive patients (Lechelt et al., 2007) also is thought to be caused by mitochondrial dysfunction (Brinkman et al., 1999; Kakuda et al., 1999), resulting from the increased rate of lipolysis induced by NRTI administration (Hadigan et al., 2002) and the adverse effects of NRTI-mediated mitochondrial dysfunction on adipocyte functioning (Brinkman et al., 1999; Kakuda et al., 1999). The effects of NRTIs on mitochondrial function may be caused by incorporation of NRTIs into mitochondrial DNA (Dagan et al., 2002; Kakuda, 2000), impairment of mitochondrial enzymes and triggering of mitochondrial-induced apoptosis (Kakuda, 2000). The effects of NRTIs on mitochondrial function have been examined in isolated mitochondria and *in vivo* and are summarised in Table 3.

Table 3: Effects of NRTIs on mitochondrial function in cell culture and animal studies

NRTI	Effect	Reference
Didanosine	Decreased mitochondrial membrane potential difference[*]	Keswani et al., 2003a
Stavudine	Decreased mitochondrial DNA content[*]	Kakuda, 2000
	Decreased calcium loading capacity[*]	Lund & Wallace, 2004
	Decreased mitochondrial membrane potential difference[*]	Keswani et al., 2003a
Zalcitabine	Changes in mitochondrial structure in rats[#]	Feldman & Anderson, 1994
	Decreased mitochondrial membrane potential difference[*]	Keswani et al., 2003a
Zidovudine	Increased calcium loading capacity[*]	Lund & Wallace, 2004
	Increased protein oxidation, release of cytochrome *c*, decreased Bcl-2, increased caspase-3 and Bax[*]	Opii et al., 2007
	Uncoupling of oxidative phosphorylation[*]	Keilbaugh et al., 1997
	Reduction in mitochondrial DNA in rats[#]	Collins et al., 2004

[*] indicates cell culture studies. [#] indicates *in vivo* studies.

1.5.1 Effects of NRTIs on mitochondrial function *in vitro*

The effects of NRTIs on the function of isolated mitochondria are dose-dependent and complex, involving various pathways, with subtle differences in the properties of specific NRTIs (Lund & Wallace, 2004). One of the ways in which NRTIs may disrupt mitochondrial activity is by decreasing mitochondrial DNA content, which may be caused by inhibition of DNA polymerase-γ activity (Birkus et al., 2002; Brinkman et al., 1999; Dalakas et al., 2001; Kakuda, 2000; Lewis & Dalakas, 1995; Lund & Wallace, 2004; Martin et al., 1994; Morris & Carr, 1999). NRTIs also may increase mitochondrial DNA mutations (Lewis, 2003). Mitochondrial DNA mutation and depletion has adverse effects on mitochondrial structure and function (Lewis, 2003). Although most NRTIs are thought to decrease mitochondrial DNA content, results from studies examining mitochondrial DNA depletion after NRTI exposure are inconsistent. In one study stavudine, unlike other commonly used NRTIs, did not cause DNA depletion of isolated mitochondria (Cui et al., 1997), while, in another study, stavudine did decrease mitochondrial DNA content (Kakuda, 2000). Also, while stavudine decreased the calcium loading capacity of isolated rat heart mitochondria, zidovudine increased calcium loading capacity (Lund & Wallace, 2004). These contradictory results indicate that the mechanisms of NRTI-induced mitochondrial toxicity are complex, and different NRTIs interfere with mitochondrial function through discrete mechanisms (Cui et al., 1997), making it difficult to find appropriate treatments for the adverse effects caused by NRTI-induced mitochondrial dysfunction in HIV-positive patients.

NRTIs also induce oxidative stress in isolated mitochondria (Opii et al., 2007) by the release of free radicals, which may cause mitochondrial DNA mutations (Kakuda, 2000) and NRTI-induced neuronal death (Opii et al., 2007). These effects may be reversed by pre-treatment with anti-oxidants (Opii et al., 2007). Zidovudine increased oxidative stress in isolated mitochondria by increasing protein oxidation and elevating the release of cytochrome *c* (Opii et al., 2007). The adverse effects of NRTIs on isolated nerve fibres (see section 1.6.1) and NRTI-induced decreases in peripheral nerve fibre density in animal models (see section 1.6.2) may be explained by what is known about the drugs' effects on isolated mitochondria. Zidovudine decreased levels of the anti-apoptotic protein Bcl-2 and increased levels of the pro-apoptotic proteins caspase-3 and Bax (Opii et al., 2007), increasing the likelihood of NRTI-induced neuronal apoptosis.

A further way in which NRTIs may impair mitochondrial function is by altering mitochondrial membrane potential difference (Keswani et al., 2003a). Zalcitabine, didanosine and stavudine, incubated with dorsal root ganglion neurones and Schwann cells, reduced neuronal mitochondrial membrane potential difference within four hours of exposure in a dose-dependent manner (Keswani et al., 2003a). Zalcitabine had the greatest effect on neuronal mitochondrial membrane potential difference, but did not cause mitochondrial depolarisation in Schwann cells. Stavudine altered mitochondrial membrane potential difference to a lesser degree than zalcitabine and didanosine, but zidovudine had no effect on membrane potential difference. Application of the immunophilin ligand FK506, which has

neuroprotective and neurotrophic properties, prevented the alterations in mitochondrial membrane potential difference induced by NRTI exposure. However, the use of immunophilins in the treatment of toxic neuropathy in HIV-positive patients is limited, because of the immunosuppressant properties of these agents (Keswani et al., 2003a).

1.5.2 Effects of NRTIs on mitochondrial function *in vivo*

Oral administration of zidovudine to rats at a dose of 100 $mg.kg^{-1}$, which falls outside the equivalent human therapeutic dose range (50-75 $mg.kg^{-1}$ per day), resulted in a reduction in mitochondrial DNA, which the authors contributed to a decrease in mitochondrial synthesis and biogenesis (Collins et al., 2004). A very low dose of zidovudine (15 $mg.kg^{-1}$ per day) had no effect on mitochondrial synthesis. The number of abnormal mitochondria also increased after NRTI administration (Dagan et al., 2002; Feldman & Anderson, 1994). Oral administration of zalcitabine to rats for up to 24 weeks resulted in time-dependent and site-specific changes in mitochondrial structure (Feldman & Anderson, 1994). Mitochondria in the sciatic nerve, tibial nerve and dorsal root ganglia were abnormally shaped and closely packed. The occurrence of these abnormal mitochondria frequently was correlated with myelin splitting associated with zalcitabine treatment (see section 1.6.2), but mitochondrial alterations appeared to precede myelin splitting (Feldman & Anderson, 1994). While zalcitabine induced morphological changes in mitochondria, there were no signs of structural damage to the mitochondria as a whole or to the cristae. The authors suggested that the

observed morphological changes occur to improve the impaired mitochondrial function caused by zalcitabine, such as changing the shape of the mitochondria to increase the surface area available for energy production (Feldman & Anderson, 1994). It appears however that these morphological changes cannot sustain normal mitochondrial function indefinitely, as these mitochondrial alterations later were followed by abnormalities in the myelin sheath of affected nerves (see section 1.6.2), which may have been caused by abnormal mitochondrial and cell functioning.

While much is known about mitochondrial dysfunction caused by NRTIs, it is poorly understood how this mitochondrial toxicity causes the nerve damage evident in peripheral neuropathy, and especially what determines whether the neuropathy is painful or not. As both the virus and antiretroviral drugs may cause neuropathy in HIV-positive patients it is difficult to separate the effects of the virus and the drugs in these patients. Thus it is necessary to examine NRTI-induced nerve damage in the absence of HIV infection, in cell culture and in animal models, to improve our understanding of toxic neuropathy. The effects of NRTIs on nerve fibres are summarised in Table 4.

Table 4: Effects of NRTIs on nerve fibres in cell culture and animal studies

NRTI	Effect	Reference
Didanosine	Apoptosis in up to 60 % of fibres*	Bodner et al., 2004
	Decreased neurite regeneration*	Cui et al., 1997
	Altered neurone morphology*	Keswani et al., 2003a
	Swelling, axon shrinkage and myelin splitting in the sciatic nerve of rats#	Schmued et al., 1996
Stavudine	Apoptosis in up to 25 % of fibres*	Bodner et al., 2004
	Decreased neurite regeneration*	Cui et al., 1997
	Altered neurone morphology*	Keswani et al., 2003a
Zalcitabine	Apoptosis in up to 28 % of fibres*	Bodner et al., 2004
	Axonal degeneration, cell death in 50 % of neurones*	Keswani et al., 2004
	Decreased neurite regeneration, decreased cell proliferation*	Cui et al., 1997
	Altered neurone morphology*	Keswani et al., 2003a
	Decreased conduction velocity, demyelination and myelin splitting in peripheral nerves of rabbits#	Anderson et al., 1992
	Increased myelin thickness and decreased cytoplasm in the sciatic nerve of rats#	Bhangoo et al., 2007
	Decreased conduction velocity of C fibres in the saphenous nerve of rats#	Chen & Levine, 2007
	Decreased epidermal nerve fibre density in the hind paw of rats#	Wallace et al., 2007b
Zidovudine	Apoptosis in up to 8 % of fibres*	Bodner et al., 2004

* indicates cell culture studies. # indicates *in vivo* studies.

1.6 Effects of NRTIs on nerve fibres

1.6.1 Effects of NRTIs on nerve fibres in cell culture

NRTIs induce axonal degeneration and apoptosis and decrease regeneration of isolated rat neurones (Bodner et al., 2004; Cui et al., 1997; Keswani et al., 2004). These effects are dose-dependent and differ in severity depending on the NRTI used.

Didanosine resulted in dose-dependent apoptosis of rat neonatal dorsal root ganglion neurones, with between 40 % and 60 % of neurones being killed by the drug (Bodner et al., 2004). This effect was enhanced with the addition of HIV glycoprotein gp120 and decreased with the addition of CEP-1347, an inhibitor of c-Jun N-terminal kinase (JNK). Thus, blocking the JNK pathway may be useful in treating HIV-related neuropathy. Zalcitabine, stavudine and zidovudine also caused apoptosis of rat dorsal root ganglion neurones, in descending order of severity (Bodner et al., 2004). The finding that didanosine had a greater adverse effect on neurone viability than zalcitabine is unusual, as zalcitabine normally is regarded as the most neurotoxic of all NRTIs.

Rat dorsal root ganglion neurones incubated for 24 hours with zalcitabine showed axonal degeneration, such that the length of neurones was decreased after the incubation period (Keswani et al., 2004). Zalcitabine also caused cell death in almost 50 % of neurones, when incubated for 36 hours. Axonal degeneration and cell death were prevented by the administration of erythropoietin, attributed to its

binding to the erythropoietin receptor on the surface of the dorsal root ganglion neurones. Although the authors failed to postulate how erythropoietin may prevent zalcitabine-induced axonal degeneration and cell death this study highlights another possible treatment for peripheral neuropathy in HIV-positive patients (Keswani et al., 2004). Administering erythropoietin to HIV-positive patients with signs of toxic neuropathy may protect nerve fibres from further nerve damage, decreasing the progression of the neuropathy and improving quality of life.

The dose-dependent neurotoxicity of therapeutic doses (1-100 µM) of NRTIs has been examined by Keswani et al. (2003a) who showed that zalcitabine, incubated with dorsal root ganglion neurones and Schwann cells for 15 hours, caused changes in neurone morphology, including varicosities in distal portions at low doses and neurite degeneration at high doses. Didanosine and stavudine had similar, but less potent, effects on neurone morphology, while no morphological abnormalities were observed following zidovudine application (Keswani et al. 2003a). These results are in keeping with the observation that zalcitabine is associated with the highest incidence of toxic neuropathy, while zidovudine does not cause peripheral neuropathy (see Table 2). Zalcitabine-induced neurotoxicity was prevented by the immunophilin FK506; however clinical application of this agent in the treatment of toxic neuropathy in HIV-positive patients is limited, because of its immunosuppressant properties (Keswani et al., 2003a).

Cui and colleagues (1997) observed the effect of various NRTIs on cell

proliferation and neurite regeneration in PC-12, a rat pheochromocytoma, an adrenal medullary tumour frequently used as an *in vitro* neuronal model. Zalcitabine, didanosine and stavudine caused a dose-dependent decrease in neurite regeneration, after neurite removal, while zidovudine and lamivudine had no effect (Cui et al., 1997). Zalcitabine also decreased cell proliferation, reducing the number of viable cells after incubation. A zalcitabine concentration of 25 µM completely inhibited cell proliferation, while didanosine, stavudine, zidovudine and lamivudine had no effect on cell proliferation (Cui et al., 1997). Moreover, stavudine did not decrease mitochondrial DNA content, unlike the other NRTIs, indicating a different mechanism of action for stavudine's effects on nerve fibres (Cui et al., 1997). The findings of Cui et al. (1997) indicate that, while zalcitabine is highly toxic to isolated neurones, which is consistent with the high incidence of toxic neuropathy associated with the use of this NRTI in HIV-positive patients (see section 1.3), zidovudine had few adverse effects on neurone viability, which may explain why zidovudine is the only NRTI not associated with peripheral neuropathy in HIV-positive patients (see section 1.3). Zidovudine did however increase markers of protein oxidation in isolated synaptosomes, which are studied as an indicator of neuronal synaptic function at nerve terminals (Opii et al., 2007), again indicating that antioxidant supplementation may be useful in the treatment of toxic neuropathy in HIV-positive patients. The finding that zidovudine adversely affected synaptosome functioning shows that, although zidovudine generally is found to be less toxic than zalcitabine and is not associated with the same degree of cell death of isolated neurones as other NRTIs, zidovudine still may adversely affect neurone

function. Increasing protein oxidation and inducing oxidative stress may have adverse effects on normal synaptic functioning (Opii et al., 2007), which may contribute to some of the side effects resulting from zidovudine treatment in HIV-positive patients (see Table 2).

In another study examining the effects of NRTIs on PC-12 Keilbaugh et al. (1997) showed that zalcitabine caused uncoupling of oxidative phoshorylation in these cells, as seen by a dose-dependent increase in lactate production and a concomitant increase in oxygen consumption. Both effects only were evident several days after the cells were incubated with the NRTI, demonstrating that zalcitabine does not directly cause uncoupling of the electron transport chain. The authors suggested that zalcitabine decreases mitochondrial DNA replication and production, which decreases the synthesis of proteins necessary for oxidative phosphorylation and thus results in the delayed increase in lactate production and oxygen consumption observed (Keilbaugh et al., 1997), which may explain the high incidence of lactic acidosis associated with NRTI use (see Table 2).

While studies examining the effects of NRTIs on isolated neurones in cell culture have shown consistently that most NRTIs result in cell death and decrease neurite regeneration, the effects of NRTIs on nerve fibres in animal models of toxic neuropathy frequently are inconsistent with each other and with the *in vitro* evidence.

1.6.2 Effects of NRTIs on nerve fibres in animal models

Although NRTI administration is associated with decreased peripheral nerve fibre density in HIV-positive patients (Cherry et al., 2003; Pardo et al., 2001; Polydefkis et al., 2002), Warner and colleagues (1995) failed to observe signs of peripheral neurotoxicity in rabbits given oral didanosine or stavudine daily for 24 weeks. One rabbit receiving a high dose (1500 $mg.kg^{-1}$) of didanosine had to be killed after eleven weeks, after it developed signs of dehydration, a loss of appetite, and poor body positioning, which forced the researchers to decrease the dose of didanosine to 1000 $mg.kg^{-1}$ for the other rabbits receiving the 1500 $mg.kg^{-1}$ dose for the remainder of the study. Even these near-lethal doses of didanosine and stavudine were not sufficient to induce significant changes in the peripheral nervous system (Warner et al., 1995). Throughout the study, plasma levels of didanosine and stavudine confirmed systemic exposure to these NRTIs, but Warner et al. (1995) found no change in peripheral nerve conduction or in the histopathology of peripheral or central nerves. Similarly, daily oral administration of stavudine for one year did not result in signs of peripheral neuropathy in monkeys (Kaul et al., 1999), although, the authors do not explain clearly how peripheral neuropathy was measured, and so these findings are difficult to interpret.

In contrast to the lack of neurotoxicity observed in rabbits after didanosine and stavudine administration (Warner et al., 1995), Anderson and colleagues (1992) found that chronic oral administration of zalcitabine induced a decrease in conduction velocity in the distal sural nerve and structural damage to neurones in

the sciatic nerve of rabbits. Neurone pathology was evident in dorsal root ganglia, the peripheral sensory system, in the ventral roots and the peripheral motor system. Several rabbits in this study exhibited clinical symptoms of neurologic damage, such as hind limb paresis and dysaesthesia, as well as gait and postural abnormalities. The severity of neural damage was dependent on the dose and the duration of drug exposure. Peripheral nerve abnormalities included demyelination and myelin splitting with intramyelinic oedema and axon shrinkage; however, some neurones exhibited signs of remyelination. These effects were more pronounced in larger diameter axons (Feldman et al., 1992) and resulted in axonal loss in peripheral nerves (Anderson et al., 1992). Inflammatory cell infiltrates were minimal and neural damage was not evident in the spinal cord or brain. The authors suggested that the morphological changes observed in peripheral neurones may be caused by impaired Schwann cell activity, resulting in abnormal myelin production (Feldman et al., 1992).

In a similar study in rats, 20 weeks of twice daily oral administration of didanosine caused morphological changes to neurones in the sciatic nerve (Schmued et al., 1996), which is in contrast to the findings of Warner at al. (1995), who showed no changes in peripheral nerve morphology following once daily didanosine administration to rabbits for 24 weeks. Schmued and colleagues (1996) showed that, in rats administered didanosine, nerve fibres were swollen, while axons were shrunken and the myelin was split into two distinct sheaths, features identical to those described following zalcitabine administration to rabbits (Feldman et al.,

1992). Subsequent studies showed that these changes first were evident after 15 weeks of daily drug administration, while abnormal nerve fibre morphology was less frequent after 20 weeks (Patterson et al., 2000). The authors suggested that the nerves may be able to adapt to the toxic effects of didanosine, making partial recovery during drug administration possible (Patterson et al., 2000), resulting in the improvement in nerve fibre morphology observed after 20 weeks. While such a recovery may be feasible in uninfected rats, it is doubtful that this process occurs in HIV-positive patients, as toxic neuropathy normally does not spontaneously disappear during continued administration of the causative agent, possibly because of the underlying neuropathy caused by the virus.

Recently, Bhangoo and colleagues (2007) found that a single intraperitoneal injection of 25 $mg.kg^{-1}$ zalcitabine to rats resulted in structural changes in the sciatic nerve similar to those observed following repeated administration of NRTIs to rabbits and rats (Anderson et al., 1992; Feldman et al., 1992; Schmued et al., 1996). While the diameter of the neurones remained unchanged, the myelin sheath was distorted and swollen, such that the cytoplasm of the neurones was decreased compared to controls. These structural changes were not observed in dorsal root ganglion neurones (Bhangoo et al., 2007). A single intravenous injection of zalcitabine also decreased the conduction velocity of C fibres in the saphenous nerve of rats, while the firing rate remained unchanged (Chen & Levine, 2007). The mechanism by which NRTI administration alters conduction velocity remains unclear.

In contrast to studies that have shown significant alterations to peripheral neurones following a single injection of zalcitabine to rats, Siau et al. (2006) found that a single injection of zalcitabine into the tail vein of rats had no effect on intra-epidermal nerve fibre density in the hind paw and did not cause activation of Langerhans cells, which contribute to epidermal inflammation by releasing pro-inflammatory cytokines and nitric oxide when activated (Siau et al., 2006). Systemic injections of zalcitabine three times a week for three weeks did however decrease epidermal nerve fibre density in the lateral plantar surface of the hind paw of rats (Wallace et al., 2007b). It appears that a single injection of an NRTI may cause morphological changes in peripheral neurones and may result in a heightened sensitivity to noxious stimulation (see section 1.7), but that a reduction in peripheral nerve fibre density occurs only with continuous NRTI administration. This finding corresponds to the observation that symptomatic toxic neuropathy in HIV-positive patients arises a minimum of one week, usually six to eight weeks or up to six months, after starting antiretroviral therapy (Husstedt et al., 2001).

Although numerous studies have examined the effects of NRTIs on peripheral nerve fibre density and morphology, few researchers have addressed the consequences of the observed nerve damage on motor function in animals. Joseph and colleagues (2004) showed that 50 $mg.kg^{-1}$ zalcitabine, administered once intravenously into the tail vein, resulted in hyperalgesia (see section 1.7) but did not significantly affect motor function or co-ordination of rats, as tested on a rotarod. In contrast to Joseph et al. (2004), Morse and colleagues (1997) found that a single oral administration of

zalcitabine resulted in a dose- and time-dependent decrease in open-field locomotor activity of rats, while oral administration of zidovudine had no effect on locomotion (Morse et al., 1997). Although different testing methods were used, the studies of Joseph et al. (2004) and Morse et al. (1997) appear to be contradictory and the effects of NRTI administration on motor function remain unclear. It also is uncertain whether the changes in peripheral neurone morphology and peripheral nerve fibre density associated with repeated NRTI administration (Anderson et al., 1992; Feldman et al., 1992; Patterson et al., 2000; Schmued et al., 1996; Wallace et al., 2007b) alter motor function in animals, as the effect of repeated NRTI administration on motor function only has been examined in one study. Wallace and colleagues (2007b) showed that, although thigmotaxis (anxiety-like behaviour) was significantly increased in zalcitabine-treated rats compared to controls at the time of peak mechanical hypersensitivity (see section 1.7), repeated systemic injections of zalcitabine to rats did not affect the total distance covered in a novel environment, indicating that zalcitabine did not cause obvious motor deficits (Wallace et al., 2007b). The findings of Wallace et al. (2007b) are in contrast to those of Morse and colleagues (1997) who showed a significant decrease in the open-field locomotor activity of rats after a single injection of zalcitabine.

The inconsistencies in the effects of NRTIs on nerve fibres and motor function in animal models may be caused by the difference in the toxicity of the NRTIs tested, by the different routes of administration employed, the duration of NRTI exposure and the different testing methods used, as well as by the animal species used. Also,

developing a robust animal model of NRTI-induced neuropathy may be of limited value because HIV infection may be necessary for toxic neuropathy to develop in HIV-positive patients (Keswani et al., 2002). The effects of NRTIs on animals, in the absence of HIV-infection, also may be minimal and varied, resulting in findings that sometimes are contradictory. Thus, because of the lack of robust animal models of the disease process, the mechanisms of toxic neuropathy are not well understood. Also, few studies have examined the effects of NRTI administration on pain sensitivity in animals, and most of these studies focused on the effects of zalcitabine, which no longer is recommended for the treatment of HIV infection, making it difficult to find appropriate treatments for the pain caused by toxic neuropathy in HIV-positive patients.

1.7 Animal models of NRTI-induced pain hypersensitivity

In one of the first animal studies focusing on pain caused by antiretroviral drugs, Joseph and colleagues (2004) showed that a single intravenous injection of the NRTIs didanosine, zalcitabine and stavudine to rats resulted in a dose-dependent mechanical and thermal hyperalgesia of the hind paw that lasted for twenty days. Hyperalgesia was observed with all of the agents within one day when a dose of 50 $mg.kg^{-1}$ was used, while injections of 25 $mg.kg^{-1}$ and 10 $mg.kg^{-1}$ resulted in hyperalgesia within three days. Blocking protein kinase and nitric oxide synthase, which has been shown to decrease hypersensitivity in other models of neuropathic pain, did not attenuate the hyperalgesia elicited by NRTI injection. Buffering intracellular calcium significantly decreased NRTI-induced mechanical

hyperalgesia. The authors suggested that calcium signalling plays a role in the development of NRTI-induced hypersensitivity and that calcium homeostasis is impaired by mitochondrial dysfunction caused by NRTIs (Joseph et al., 2004).

Subsequent studies showed that disrupting the mitochondrial electron transport chain and preventing mitochondrial phosphorylation, which is useful in treating other types of neuropathic pain, attenuated zalcitabine-induced hyperalgesia and allodynia (Joseph & Levine, 2006). The authors concluded that pathways dependent on the mitochondrial electron transport chain, particularly those in primary afferent nociceptors, may play a role in NRTI-induced neuropathic pain. This suggestion however is not in agreement with the proposed mechanism of action of NRTIs, which assumes that NRTIs cause inhibition of mitochondrial DNA synthesis and a subsequent decrease in the production of mitochondrial proteins, which results in a decrease in mitochondrial phosphorylation (see section 1.5). Thus, administering agents that block the mitochondrial electron transport chain might be expected to exacerbate the effects of NRTIs and possibly enhance the hyperalgesia caused by NRTIs, instead of abolishing NRTI-induced hyperalgesia as shown by Joseph and Levine (2006). These findings highlight the complexity of NRTI-induced neuropathic pain and further studies examining the role of the electron transport chain in NRTI-mediated hypersensitivity are necessary to clarify these results.

Recently Wallace et al. (2007b) found that zalcitabine, injected intraperitoneally three times a week for three weeks, induced mechanical hyperalgesia and anxiety-

like behaviour in rats and decreased epidermal nerve fibre density (see section 1.6.2), but did not affect heat or cold sensitivity. The finding that zalcitabine administration did not alter heat or cold sensitivity contradicts the results of Joseph et al. (2004) who showed that a single intravenous injection of zalcitabine induced thermal hyperalgesia in rats. Also, most HIV-positive patients with peripheral neuropathy present with impaired thresholds for hot and cold stimulation and this criterion frequently is used to diagnose peripheral neuropathy in these patients (Cherry et al., 2005; Huengsberg et al., 1998; Martin et al., 2003; McArthur et al., 2005). Thus, although animal models of NRTI-related neuropathy can help improve our understanding of the mechanisms of the disease, it is necessary to interpret the results of these studies with caution, when translating the effects of NRTI administration in animals to the effects of NRTI administration in HIV-positive patients.

Although systemic administration of zalcitabine to rats did not alter heat and cold sensitivity, Wallace and colleagues (2007b) did show that mechanical hyperalgesia occurred throughout the study. Mechanical hyperalgesia was observed from six days after the first injection, when doses of 50 $mg.kg^{-1}$ and 25 $mg.kg^{-1}$ were used, while injections of 5 $mg.kg^{-1}$ did not change the mechanical threshold at any time. Zalcitabine injection did not result in the expression of stress-related factors in dorsal root ganglion neurones, but enhanced expression of the chemokine CCL2, indicating that chemokines play a role in NRTI-induced pain hypersensitivity. Zalcitabine injection also caused an increase in macrophage infiltration in dorsal

root ganglion neurones, but macrophage infiltration was not evident in peripheral neurones. Systemic zalcitabine administration caused only modest increases in astrocyte and microglial activity in the spinal dorsal horn, and blocking microglial activity by concomitant administration of minocycline did not alter zalcitabine-induced hyperalgesia (Wallace et al., 2007b). The authors concluded that zalcitabine has a limited effect on primary sensory neurones and spinal afferent pathways and that further studies are required to elucidate the mechanisms by which these drugs induce neuropathic pain.

In another study, Siau et al. (2006) found that a single intravenous injection of zalcitabine to rats induced mechanical hypersensitivity, but did not cause a decrease in peripheral nerve fibre density (see section 1.6.2). More recently Bhangoo and colleagues (2007) showed that a single intraperitoneal injection of 25 $mg.kg^{-1}$ zalcitabine resulted in a decrease in the paw withdrawal threshold of rats for 42 days from day three after injection, but the rats did not exhibit changes in grooming behaviour or appearance. Blocking the CXCR4 chemokine receptor, which plays a role in gp120-mediated neurotoxicity (see section 1.2), abolished the allodynia induced by zalcitabine injection. The results of this study and those of Wallace and colleagues (2007b) show that, just as chemokines are implicated in the heightened pain sensitivity caused by gp120 administration (see section 1.2), chemokines also may be involved in the production of NRTI-induced pain.

While the injection models of NRTI-induced neuropathy have yielded valuable

insights into the development of NRTI-induced pain, these models may not be entirely suitable to examine the underlying mechanisms of this pain, as antiretroviral drugs are administered orally to HIV-positive patients, and neuropathy in these patients normally develops only after six to eight weeks of chronic NRTI administration (Husstedt et al., 2001). Joseph and colleagues (2004) however did also show that daily oral administration of the NRTI zalcitabine to rats at a dose of 50 $mg.kg^{-1}$ for six weeks resulted in mechanical and thermal hyperalgesia in the hind paw from seven days onward. A dose of 25 $mg.kg^{-1}$ of zalcitabine induced hyperalgesia only after three weeks of daily oral administration.

These rat models of NRTI-induced pain hypersensitivity show that the dose and the route of administration are determinants of the hypersensitivity caused by NRTIs. While intravenous administration of the causative agent results in hyperalgesia and allodynia one day after injection (Joseph et al., 2004; Siau et al., 2006), hypersensitivity develops more slowly following systemic administration, occurring between three and six days after injection (Bhangoo et al., 2007; Wallace et al., 2007b). Although the oral bioavailabilty of NRTIs is high (Kaul et al., 1999; Kelley et al., 1987), hypersensitivity only was evident after one week of daily oral administration of 50 $mg.kg^{-1}$ zalcitabine to rats (Joseph et al., 2004). However, the occurrence of hyperalgesia and allodynia after seven days of daily oral NRTI administration in rats still is much faster than the development of painful peripheral neuropathy in HIV-positive patients, which usually occurs only after six weeks of antiretroviral therapy (Husstedt et al., 2001).

It is important to investigate the effects of NRTIs on pain hypersensitivity in animals, to determine possible mechanisms of the pain induced by these drugs in HIV-positive patients, but both the virus and the antiretroviral drugs are associated with the development of painful peripheral neuropathy in HIV-positive patients, and thus it also is useful to examine the combined effects of NRTI and gp120 administration on pain sensitivity in animal models. To my knowledge only two such studies exist, and both show that most of the effects of the virus on neurone functioning are enhanced by NRTI exposure.

1.8 Interaction of gp120 and NRTIs

Keswani et al. (2006) administered 25 $mg.kg^{-1}$ didanosine daily to gp120 transgenic mice, which express gp120 in astrocytes, for four weeks. Didanosine was dissolved in the drinking water and the spontaneous expression of gp120 in the sciatic nerve and spinal cord was confirmed at the start of the study. Intra-epidermal nerve fibre density was decreased after four weeks in transgenic mice administered didanosine, with the greatest decrease occurring in unmyelinated fibres (Keswani et al., 2006). Neither gp120 expression without didanosine exposure, nor didanosine administration to control mice affected epidermal nerve fibre density (Keswani et al., 2006), indicating that both the virus and NRTI exposure may indeed be necessary for neuropathy to develop in HIV-positive patients (Keswani et al., 2002). Similarly, only transgenic mice receiving didanosine developed mild thermal hyperalgesia, with no further differences in behavioural or electrophysiological measures between groups. Sciatic nerve morphology also was identical in all mice.

Keswani and colleagues (2006) concluded that the results of their study correlate with clinical findings in HIV-positive patients with early sensory neuropathy and suggested that this model could be used to further examine the mechanisms of, and develop treatments for, peripheral neuropathy in HIV infection.

Although Keswani and colleagues (2006) found that the combined effect of gp120 and didanosine altered peripheral nerve fibre density and caused hyperalgesia in mice, mice expressing gp120 without receiving didanosine did not exhibit the vast changes in pain sensitivity and the reduction in epidermal nerve fibre density observed with exogenous gp120 administration to rodents without concomitant NRTI administration (see section 1.2). While the authors stated that spontaneous expression of gp120 was measured in transgenic mice during the study, Keswani et al. (2006) failed to give an indication of the amount of gp120 expressed and how this measure compares to the amount of exogenous gp120 administered to rodents in other studies. Thus, it is possible that the spontaneous expression of gp120 in transgenic mice was minimal, and insufficient to induce hyperalgesia and nerve damage without the added adverse effects of didanosine.

Besides finding that hyperalgesia did not develop in gp120 transgenic mice without concomitant didanosine administration, Keswani et al. (2006) also showed that hyperalgesia did not occur with daily oral administration of 25 $mg.kg^{-1}$ didanosine to control mice for four weeks. This finding is in contrast to the observations of Joseph et al. (2004), who showed that a single intravenous injection of 25 $mg.kg^{-1}$

didanosine caused hyperalgesia in rats within three days and that this hyperalgesia persisted for the twenty days of the study. The difference between the two studies may be explained by the different routes of administration employed and may be clarified by the different effects of oral and intravenous zalcitabine administration on pain hypersensitivity in rats. Although Joseph and colleagues (2004) found that both oral and intravenous administration of zalcitabine to rats caused hyperalgesia, the hyperalgesia induced by oral administration of zalcitabine arose later and was of a lesser intensity than that caused by intravenous injection of the same agent. Thus, oral administration of NRTIs appears to have fewer toxic effects than intravenous administration, which may explain why a single intravenous injection of didanosine resulted in hyperalgesia in rats (Joseph et al., 2004), while oral administration of didanosine, which is less neurotoxic than zalcitabine (Dalakas, 2001), did not change pain behaviours in mice over four weeks (Keswani et al., 2006).

In a more extensive study examining the interaction of gp120 and NRTIs, exogenous gp120 was administered directly to the sciatic nerve of rats via cellulose wrapped lightly around the nerve (Wallace et al., 2007b). Zalcitabine (50 $mg.kg^{-1}$) was injected intraperitoneally at the time of surgery and three times a week for three weeks thereafter. The simultaneous administration of both agents resulted in more pronounced mechanical hyperalgesia, a greater decrease in epidermal nerve fibre density and a greater increase in the expression of the chemokine CCL2 than induced by either treatment alone (see sections 1.2, 1.6.2 and 1.7). Zalcitabine also enhanced the microgliosis induced by gp120 administration (see section 1.2), but

had no effect on gp120-mediated astrocytosis. Blocking microglial activity by concomitant administration of minocycline delayed the onset of the mechanical hyperalgesia caused by the combination of gp120 and zalcitabine, while morphine completely resolved the hyperalgesia induced by the simultaneous administration of both agents. These results indicate that HIV and NRTIs cause peripheral neuropathy and pain by different mechanisms and that the effects of these agents frequently are synergistic (Wallace et al., 2007b). This study also strengthens the theory that NRTIs enhance the adverse effects of the virus in HIV-positive patients (Keswani et al., 2002).

1.9 Thesis aims

Although zalcitabine frequently is used in animal and cell culture studies to examine the adverse effects of NRTIs, results of these studies are of limited value to HIV-positive patients, as zalcitabine no longer is recommended for the treatment of HIV infection, because of the high incidence of peripheral neuropathy associated with the use of this NRTI. Until recently stavudine was recommended by the World Health Organisation (WHO) as part of first-line antiretroviral drug regimens (WHO, 2004), but since the introduction of newer, less toxic antiretroviral drugs stavudine seldom is administered to HIV-positive patients in first-world countries. Currently, stavudine still is prescribed regularly to HIV-positive patients in developing countries, as only a limited variety of antiretroviral drugs is available in these regions. In South Africa stavudine is recommended by the National Antiretroviral Treatment Guidelines as the backbone of both available first-line antiretroviral

treatment regimens for HIV-positive adults in the public sector, as well as the recommended regimens for children infected with HIV (Grimwood, 2004). Stavudine is included in first-line antiretroviral treatment regimens, because combinations of antiretroviral drugs that include stavudine produce greater increases in CD4 cell count than do other antiretroviral drug combinations not including stavudine (Mocroft et al., 2006), which has long-term health benefits for HIV-positive patients. If the initial drug regimen was unsuccessful or intolerable didanosine is prescribed as part of second-line antiretroviral therapy. Although the NRTI lamivudine also is prescribed as part of first-line regimens in South Africa (Grimwood, 2004), toxic neuropathy caused by stavudine administration in particular is one of the most common reasons for patients discontinuing antiretroviral drug therapy; therefore I chose to use stavudine, and not lamivudine, in my studies.

Thus, the primary aim of my PhD was to examine how chronic oral administration of stavudine affects nociception in rats, and specifically, whether the drug induces hyperalgesia. As stavudine has been associated with other side effects such as hepatitis, pancreatitis and gastrointestinal disturbances (Montessori et al., 2004), I also wanted to examine whether long-term daily stavudine administration affects the overall condition of the rats, and, particularly, produces deficits resulting from neural malfunction. Consequently I also investigated the effect of daily stavudine administration on body mass, food intake and voluntary wheel running activity.

After establishing a rat model of stavudine-induced hyperalgesia, I wanted to investigate possible mechanisms of the hyperalgesia caused by oral stavudine administration in this model. Wallace et al. (2007b) recently showed that repeated systemic injection of zalcitabine to rats resulted in a modest increase in microglial and astrocyte activity in the dorsal horn, with only a limited effect on dorsal root ganglion phenotype. The role of spinal neuronal damage and central pro-inflammatory cytokines and chemokines in NRTI-induced neuropathy remains unexplored. As stavudine is known to cause nerve damage in vitro (Bodner et al., 2004; Cui et al., 1997; Keswani et al., 2004), and other types of neuropathic pain are associated with neuronal death in the dorsal horn (Scholz et al., 2005), I examined whether oral administration of stavudine induces apoptosis or necrosis of spinal neurones in rats. Secondly, as pro-inflammatory cytokines in the spinal cord are involved in the development of other types of neuropathic pain (DeLeo et al., 1996; DeLeo et al., 1997; Murphy et al., 1995; Ohtori et al., 2004; Wieseler-Frank et al., 2005), and possibly NRTI-induced pain (Pardo et al., 2001), I investigated whether daily oral administration of stavudine causes the release of IL-6, which is increased in the spinal cord in other rat models of neuropathic pain (DeLeo et al., 1996; Murphy et al., 1995). As chemokines also are thought to be involved in NRTI-induced neuropathy (Bhangoo et al., 2007; Wallace et al., 2007b), I investigated whether daily oral administration of stavudine causes the release of cytokine-induced neutrophil chemo-attractant (CINC)-1 in the spinal cord of rats. Intracerebroventricular injection of CINC-1 decreases the mechanical nociceptive threshold of rats (Yamamoto et al., 1998) and CINC-1 is involved in the

development of other types of pain (Loram et al., 2007a; Loram et al., 2007b) and thus may be involved in stavudine-induced hyperalgesia.

Besides causing peripheral neuropathy, stavudine administration also is associated with other adverse effects, such as lipodystrophy, the fat redistribution characterised by peripheral fat loss and central fat accumulation (Lechelt et al., 2007), and lactic acidosis caused by mitochondrial dysfunction (Montessori et al., 2004). In HIV-positive patients these side effects frequently are associated with increased plasma pro-inflammatory cytokine concentration, decreased plasma adiponectin concentration (Jones et al., 2005; Lindegaard et al., 2004) and increased plasma lactate levels (Brew et al., 2003; Geddes et al., 2006; Haugaard et al., 2005) respectively. Thus, in my third study, to investigate whether a systemic inflammatory response or metabolic dysregulation is responsible for the hypernociception induced by stavudine in rats, I determined whether plasma adiponectin, lactate, IL-6 and CINC-1 concentrations were altered in rats administered daily stavudine.

CHAPTER TWO

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Oral administration of stavudine induces hyperalgesia without affecting activity in rats

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Abstract

WEBER, J., D. MITCHELL AND P. R. KAMERMAN. Oral administration of stavudine induces hyperalgesia without affecting activity in rats. PHYSIOL BEHAV 92: 807 - 813, 2007.−We have investigated whether long-term oral administration of the nucleoside reverse transcriptase inhibitor (NRTI) stavudine affects nociception in Sprague-Dawley rats, and whether any changes of nociception are accompanied by deterioration in activity and appetite. Stavudine (50 $mg.kg^{-1}$) was administered to rats orally once daily for six weeks in gelatine cubes. Mechanical hyperalgesia of the tail was assessed using a bar algometer, and thermal hyperalgesia by tail immersion in 49 ºC water. Withdrawal latencies were compared to those of rats receiving placebo gelatine cubes. Withdrawal latencies to the noxious thermal challenge were not affected by stavudine, but those to the mechanical challenge were significantly decreased in rats receiving stavudine, compared to rats receiving placebo, from week three to week six of drug administration ($P<0.05$, ANCOVA with Newman-Keuls post-hoc comparisons). The overall condition of the rats was assessed by recording daily voluntary wheel running distance and maximum running speed, food intake and body mass. Daily stavudine administration did not adversely affect voluntary running activity, appetite or growth. We have shown that long-term daily oral administration of the NRTI stavudine results in mechanical hyperalgesia in rats within three weeks without affecting appetite, growth and physical activity.

1. Introduction

Pain is a common complaint of HIV-positive patients, even in the absence of AIDS-defining diseases, and frequently is underestimated and treated poorly by doctors [3,7,15]. HIV-related pain often is neuropathic in origin, not only because of neural damage caused by the virus, but also because antiretroviral drugs cause toxic neuropathies [4,6]. Although antiretroviral drugs effectively retard the progression of the disease, the prevalence of sensory neuropathy in HIV-positive patients has increased since the introduction of these drugs [4,27]. This increased incidence of neuropathy is particularly related to nucleoside reverse transcriptase inhibitors (NRTIs) [4,16], which form an integral part of Highly Active Antiretroviral Therapy (HAART).

NRTIs cause delayed cell doubling and decreased mitochondrial DNA content [4,17,19], possibly by inhibiting DNA polymerase-γ activity [17]. In HIV-positive patients, administration of NRTIs is associated with axonal degeneration and the loss of small unmyelinated nerve fibres, resulting in decreased peripheral nerve fibre density [4,25,26]. However, not all HIV-positive patients experience pain, even if they have other signs of peripheral neuropathy [16,18]. Our poor understanding of how the mitochondrial toxicity of NRTIs causes pain is partly because of a lack of animal models of the disease process.

In one of the few animal studies focusing on pain caused by antiretroviral drugs, Joseph and colleagues [11] showed that a single intravenous injection of the NRTIs

didanosine (ddI), zalcitabine (ddC) and stavudine (d4T) resulted in a dose-dependent hyperalgesia of the hind paw that lasted for twenty days. Subsequent studies showed that disrupting the mitochondrial electron transport chain attenuated zalcitabine-induced hyperalgesia [12]. However, as antiretroviral drugs are administered orally to HIV-positive patients, and neuropathy in these patients normally develops only after six to eight weeks of chronic NRTI administration [10], the injection model of NRTI-induced neuropathy may not be entirely suitable to examine the mechanisms of NRTI-induced pain. Joseph and colleagues [11] however did also show that daily oral administration of the NRTI zalcitabine to rats at a dose of 50 $mg.kg^{-1}$ for six weeks resulted in hyperalgesia in the hind paw after seven days.

Although zalcitabine is effective at treating HIV, stavudine is prescribed more commonly, and is recommended by the World Health Organisation (WHO) as part of first-line antiretroviral drug regimens [31]. Combinations of antiretroviral drugs that include stavudine produce greater increases in CD4 cell count than do other antiretroviral drug combinations not including stavudine [20]. In addition, while neuropathy is the most common reason for patients discontinuing the use of stavudine, stavudine is less neurotoxic than is zalcitabine [6]. Therefore, the aim of our study was to investigate how long-term daily oral administration of the NRTI stavudine affects nociception in rats. As the drug has been associated with other side effects such as hepatitis, pancreatitis and gastrointestinal disturbances [22], we also wanted to expand the work of Joseph et al. [11] by examining whether long-

term daily stavudine administration affects the overall condition of the rats, and, particularly, produces deficits resulting from neural malfunction. Consequently we also investigated the effect of daily stavudine administration on food intake and voluntary running activity.

2. Methods

2.1. Animals

Experiments were performed on female Sprague-Dawley rats that were housed individually and had free access to standard rat chow and water. All procedures were approved by the Animal Ethics Screening Committee of the University of the Witwatersrand (clearance no. 2004/20/3).

2.2. Drug administration

Stavudine (Zerit, Bristol-Myers Squibb, Johannesburg, South Africa) was administered orally once daily, at a dose of 50 $mg.kg^{-1}$, as a suspension set in a flavoured gelatine cube. Gelatine cubes were made by adding 7ml savoury bread spread (Bovril, Unilever, Johannesburg, South Africa), 20 g cane sugar and 12 g unflavoured gelatine powder (Davis Gelatine, Johannesburg, South Africa) to 100 ml warm water [13]. The solution was aliquoted into 3 ml moulds and allowed to set. Stavudine-containing gelatine cubes were made by adding powdered stavudine to each aliquot, and mixing thoroughly before the gelatine set. Placebo gelatine cubes did not contain stavudine. Rats were fed placebo gelatine cubes, once daily, for one week before the start of experimentation, by which time they ate the entire

cube within 15 minutes of it being placed in their cage. This method of administering a drug allows a precise dose to be administered orally, without the rat being handled.

2.3. Nociceptive testing

We tested for hyperalgesia by recording the withdrawal latency to a noxious mechanical challenge and a noxious thermal challenge applied to the tail of rats placed in clear plastic restrainers, which restricted trunk movement but allowed free movement of the tail. The rats were familiarized with the restrainers for three hours a day for three consecutive days before measurements began. All measurements were made by the same observer between 09:00 and 12:00 in the morning. The withdrawal latency was recorded only when the rat displayed a clear tail withdrawal from the noxious challenge or the rat tried to turn around in the restrainer to get at the noxious challenge being applied to the tail. Other nondescript end-points, such as the rat starting to fidget were ignored.

2.3.1. Noxious mechanical challenge

A bar algometer with a 1mm diameter probe (Haldex AB, Halmstad, Sweden), was placed across the dorsal surface of the middle of the tail and a static force of 4 N was applied [29]. The time taken for the rat to withdraw its tail was recorded with a stopwatch. The test was repeated three times for each rat at slightly displaced sites, with at least one minute between each measurement, and the average of the three

measurements was recorded as the withdrawal latency for each rat. The algometer was removed from the tail if the rat had not reacted after 30 s.

2.3.2. Noxious thermal challenge

The tail of each rat was submerged in 29 °C water for 30 min before testing began. Thereafter, the whole tail of each rat was submerged in 49 °C water [9]. The time taken for the rat to show a characteristic tail flick response was recorded with a stopwatch. The test was repeated three times for each rat, with at least one minute between each measurement, and the average of the three measurements was recorded as the withdrawal latency for each rat. The tail was removed from the water if the rat had not reacted after 30 s.

2.4. Voluntary activity, body mass and food intake

To assess the general health status of the rats and possible motor defects, we recorded voluntary running activity, body mass and food intake. Rats were weighed daily and food containers were filled daily with 60 g of standard pelleted rat chow. Daily food intake was measured by subtracting the amount of food remaining in the food container and on the cage floor every morning from the amount of food given the preceding day. Because we wanted to monitor whether stavudine affects voluntary exercise, we selected rats that ran spontaneously on running wheels attached to their cages. To select the rats, we recorded the distance 30 rats ran each night using odometers (Cateye Tomo XC, Cyclocomputer, Model CC-ST200) attached to the running wheels, and then selected the 20 rats that ran the furthest

over 12 consecutive nights for subsequent nociceptive testing. Running distance and maximum running speed then were measured daily for each rat for the remainder of the study.

2.5. Experimental protocol

After the 20 rats had been selected for the study, we recorded baseline values for the withdrawal latency to the noxious challenges, voluntary running activity, body mass and food intake daily for five days before the start of stavudine administration. Throughout this period, all rats were given placebo gelatine cubes once daily. On the sixth day, rats in the experimental group received gelatine cubes containing 50 $mg.kg^{-1}$ stavudine, and continued to be fed stavudine cubes once daily for six weeks (n=10). Rats in the control group continued to be fed placebo gelatine cubes once daily for six weeks (n=10). Nociceptive tests were performed once a week, commencing seven days after the first day of stavudine or placebo administration.

2.6. Data analysis

All data are expressed as mean ± SEM. The average of the withdrawal latencies measured on the last three days before stavudine or placebo administration began served as a baseline value against which changes in withdrawal latency were compared. During stavudine or placebo administration weekly maximum running speed and weekly food intake was compared to the average maximum running speed and food intake recorded five days before the start of stavudine or placebo

administration. Weekly running distance was compared to the average running distance recorded over the days before stavudine or placebo administration.

Changes in withdrawal latencies to the noxious challenges, changes in daily running distance and absolute maximum daily running speed were assessed by means of two-way Analysis of Covariance (ANCOVA) using group and time as the main effects and rat mass as covariate, with Newman-Keuls post-hoc comparisons if main effects or interaction were significant. ANCOVA was used because previous experience has shown us that as rats grow, and their tails become thicker and the skin more keratinised, their response to the noxious mechanical and thermal challenge changes. Also, voluntary wheel running changes with age in female rats [1]. Body mass and food intake, per 100 g body mass, were assessed by means of two-way Analysis of Variance (ANOVA), using group and time as the main effects, with Newman-Keuls post-hoc comparisons if main effects or interaction were significant. Initial body mass was compared to that on the day before the start of stavudine or placebo administration and on the last day of each week of drug administration. No rats reached the cut-off of 30 s in either of the two nociceptive tests and no rats lost more than 15 % of body mass during the study.

3. Results

3.1. Noxious mechanical challenge

Before stavudine or placebo administration, the withdrawal latency to the 4N mechanical challenge applied to the tail was 10.07 ± 0.83 s for rats receiving placebo gelatine cubes and 11.01 ± 0.87 s for rats receiving stavudine gelatine cubes (t-test: t=0.78, P=0.45). Compared to rats receiving placebo gelatine cubes, and compared to withdrawal latencies measured before stavudine administration, there was a significant decrease in the withdrawal latencies of rats given stavudine from week three to week six of stavudine administration (two-way ANCOVA; group effect: $F_{1,15}$=8.29, P=0.01; time effect: $F_{6,6}$=1.75, P=0.12; interaction: $F_{6,90}$=2.24, P=0.04; Figure 1a).

3.2. Noxious thermal challenge

Before stavudine or placebo administration, the withdrawal latency to the 49 ºC thermal challenge was 4.82 ± 0.32 s for rats receiving placebo gelatine cubes and 4.79 ± 0.34 s for rats receiving stavudine gelatine cubes (t-test: t=0.08, P=0.94). There was no significant difference in the change in withdrawal latencies to the noxious thermal challenge over time, nor between rats given stavudine and placebo gelatine cubes (two-way ANCOVA; group effect: $F_{1,15}$=0.36, P=0.56; time effect: $F_{6,6}$=1.43, P=0.21; interaction: $F_{6,90}$=0.79, P=0.58; Figure 1b).

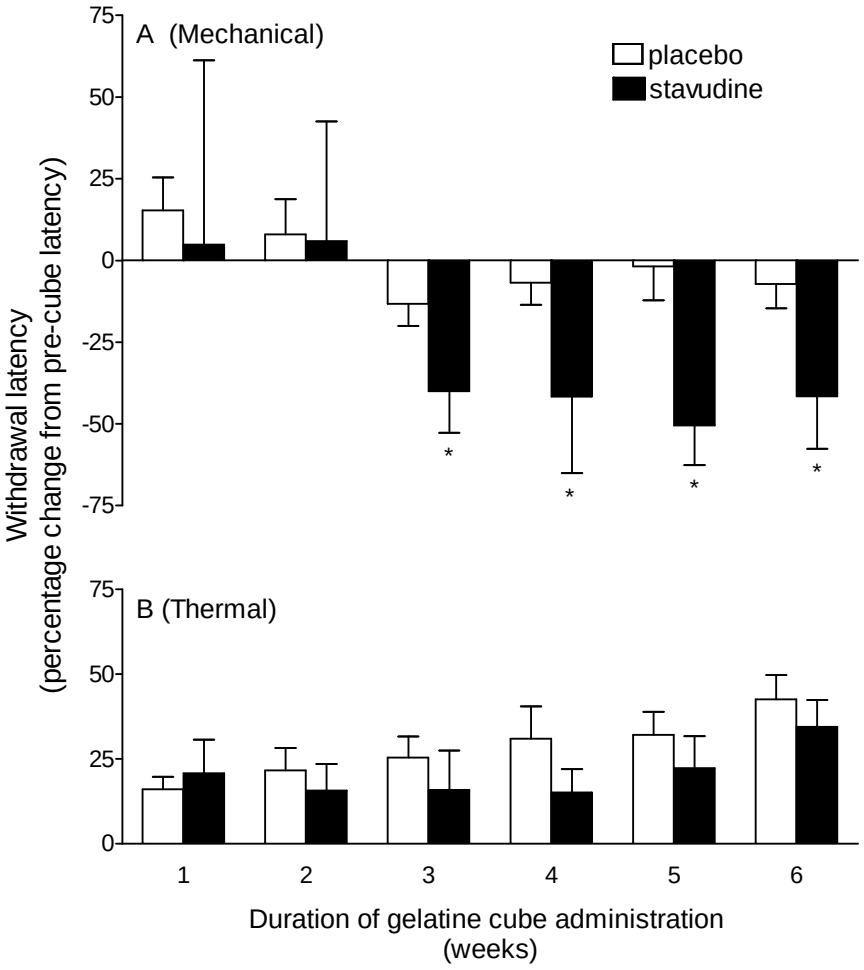

Figure 1. Changes in the withdrawal latencies (mean ± SEM) to a noxious mechanical challenge (A) and a noxious thermal challenge (B) on the tail of rats given oral placebo (clear bars) or 50mg.kg^{-1} stavudine (solid bars). Withdrawal latencies are expressed as percentage change from latencies before cube administration. * indicates a significant difference in withdrawal latencies between the two groups of rats ($P<0.05$, ANCOVA with Newman-Keuls post-hoc comparisons)

3.3. Body mass

The initial body mass of rats receiving placebo gelatine cubes was 172.6 ± 6.4 g and that of rats receiving stavudine gelatine cubes was 175.4 ± 5.3 g (t-test: t=0.34, P=0.74). There was no significant difference in body mass between rats given stavudine and placebo gelatine cubes (two-way ANOVA; group effect: $F_{1,16}$=0.36, P=0.56). Compared to the initial body mass, body mass increased significantly on each subsequent day analysed, for both groups of rats (two-way ANOVA; time effect: $F_{7,7}$=898, P<0.01; Figure 2). There was no interaction (two-way ANOVA; $F_{7,112}$=0.29, P=0.96).

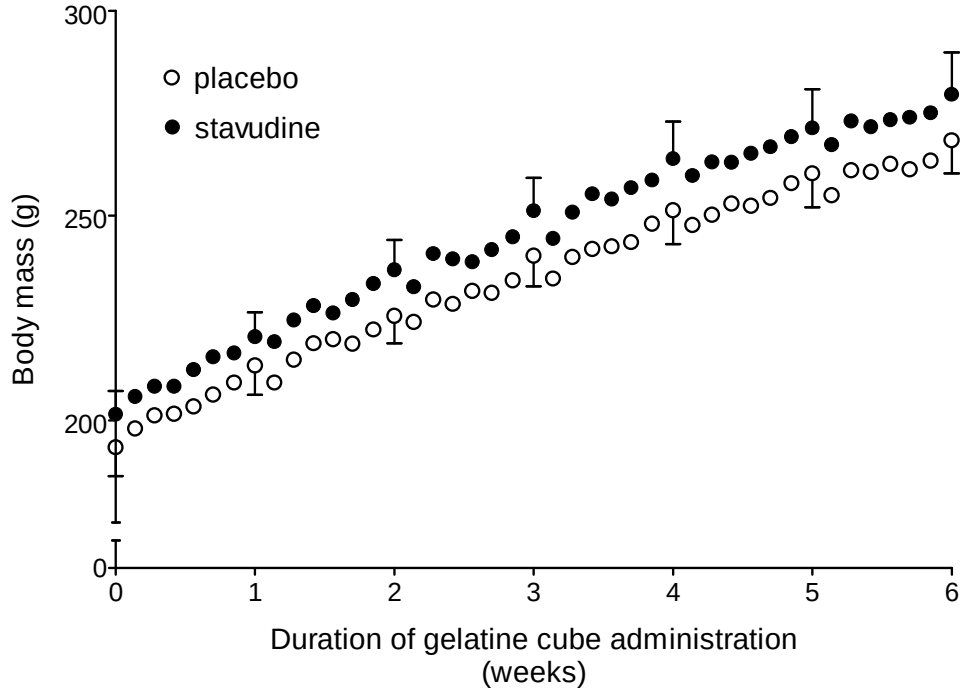

Figure 2. Body mass (mean ± SEM) of rats given oral placebo (clear circles) or 50mg.kg^{-1} stavudine (solid circles). Error bars are shown on days that were compared using statistical analysis once a week. There were no significant differences between the two groups of rats. Compared to the initial body mass, body mass increased significantly on each subsequent day analysed for both groups of rats ($P<0.05$, ANOVA with Newman-Keuls post-hoc comparisons).

3.4. Food intake

Before stavudine or placebo administration, food intake was 11.5 ± 0.4 g of food per 100 g body mass for rats receiving placebo gelatine cubes and 12.4 ± 0.5 g of food per 100 g body mass for rats receiving stavudine gelatine cubes (t-test: t=1.30, P=0.21). There was no significant difference in food intake, per 100 g body mass, between rats given stavudine and placebo gelatine cubes (two-way ANOVA: group effect: $F_{1,16}$=0.36, P=0.56). There was a significant decrease in food intake, per 100 g body mass, compared to food intake before stavudine or placebo administration, for both groups of rats from week two to week six of stavudine and placebo administration (two-way ANOVA; time effect: $F_{6,6}$=108, P<0.01; Figure 3). There was no interaction (two-way ANOVA; $F_{6,96}$=0.69, P=0.66).

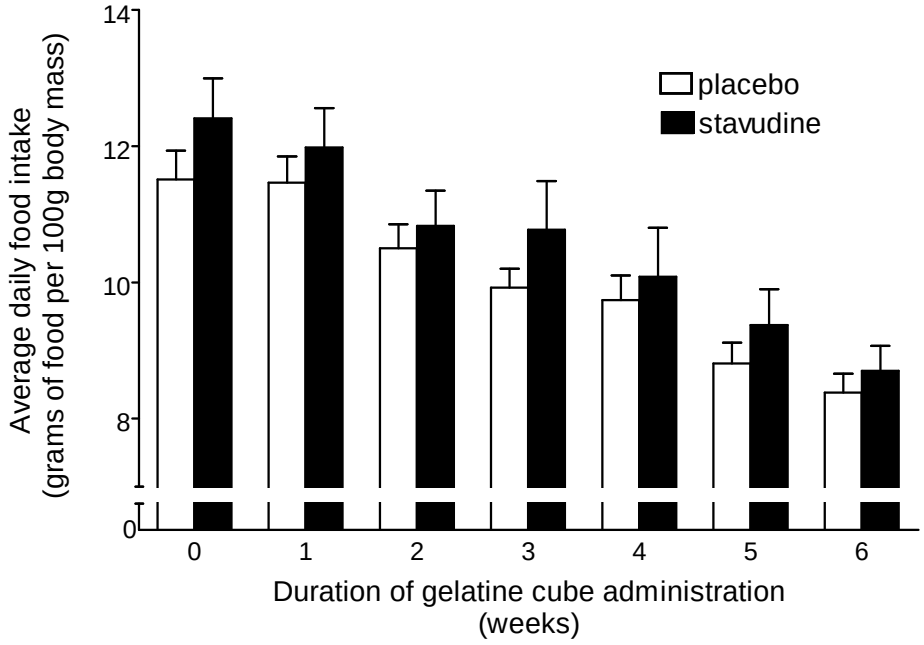

Figure 3. Food intake (mean ± SEM) of rats given oral placebo (clear bars) or 50mg.kg^{-1} stavudine (solid bars). There were no significant differences between the two groups of rats. For both groups of rats, average daily food intake, per 100g body mass, decreased from week two onward ($P<0.05$, ANOVA with Newman-Keuls post-hoc comparisons).

3.5. Voluntary running distance

Before stavudine or placebo administration, average daily running distance was 3.51 ± 0.5 km for rats receiving placebo gelatine cubes and 5.0 ± 0.7 km for rats receiving stavudine gelatine cubes (t-test: t=1.78, P=0.09). There was no significant difference in the change in running distance between rats given stavudine and placebo gelatine cubes (two-way ANCOVA; group effect: $F_{1,15}$=0.001, P=0.98). There was a significant increase in voluntary running distance, compared to that before stavudine or placebo administration, for both groups of rats from week one to week four of stavudine or placebo administration (two-way ANCOVA; time effect: $F_{6,6}$=4.52, P<0.01; Figure 4). There was no interaction (two-way ANCOVA; $F_{6,90}$=1.25, P=0.29).

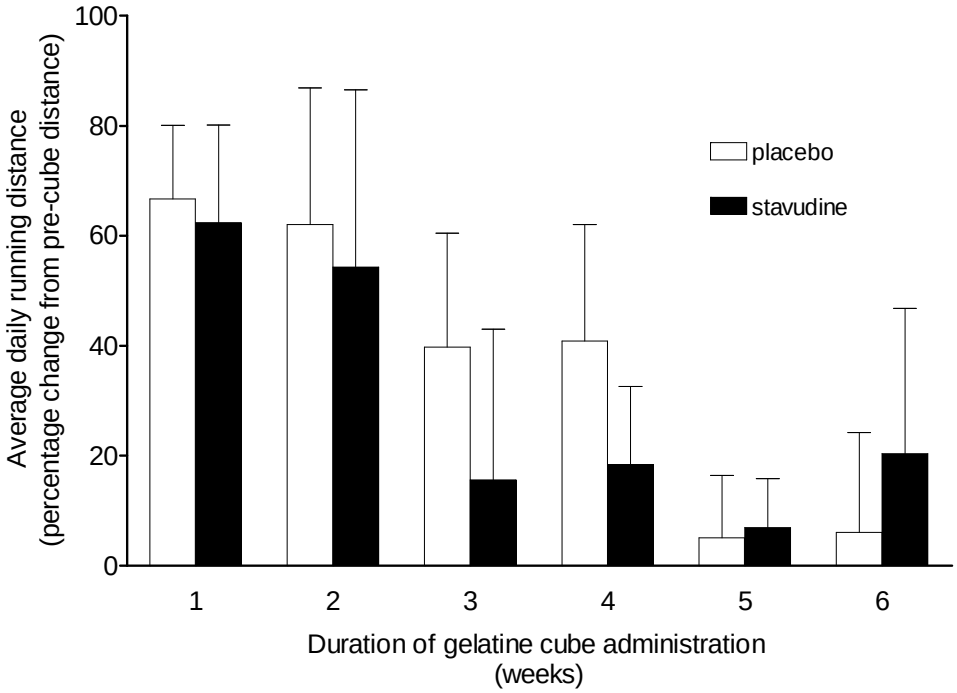

Figure 4. Changes in running distance (mean ± SEM) of rats given oral placebo (clear bars) or 50mg.kg^{-1} stavudine (solid bars). There were no significant differences between the two groups of rats. For both groups of rats voluntary running distance increased from week one to week four, but decreased to pre-cube distances by week five ($P<0.05$, ANCOVA with Newman-Keuls post-hoc comparisons).

3.6. Maximum running speed

Before stavudine or placebo administration, maximum daily running speed was 4.2 ± 0.4 $km.h^{-1}$ for rats receiving placebo gelatine cubes and 4.6 ± 0.1 $km.h^{-1}$ for rats receiving stavudine gelatine cubes (t-test: t=1.94, P=0.36). There was no significant difference in the maximum daily running speed over time, nor between rats given stavudine and placebo gelatine cubes (two-way ANCOVA; group effect: $F_{1,14}$=0.05, P=0.83; time effect: $F_{6,6}$=2.88, P=0.11; interaction: $F_{6,84}$=0.58, P=0.75; Figure 5).

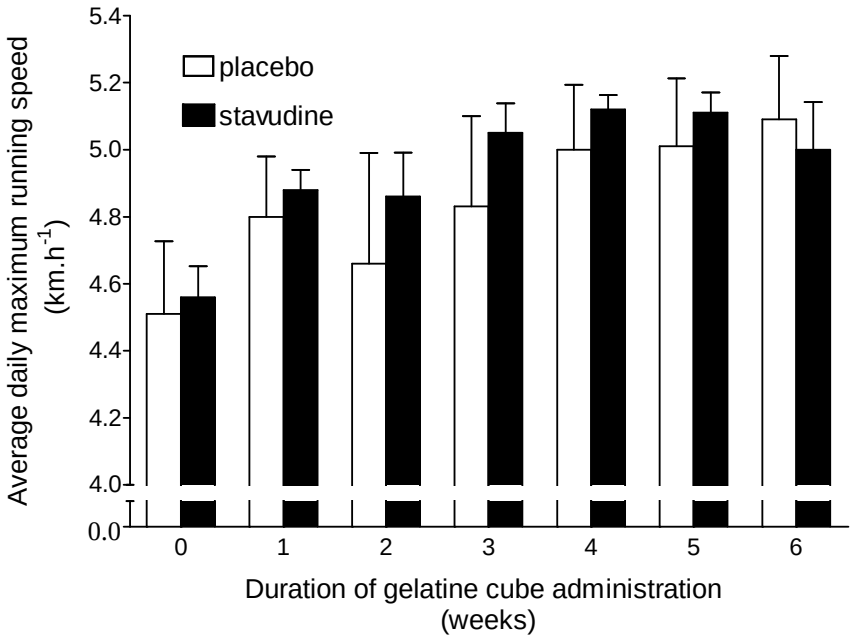

Figure 5. Maximum daily running speed (mean ± SEM) of rats given oral placebo (clear bars) or 50mg.kg^{-1} stavudine (solid bars). There were no significant differences between the two groups of rats.

4. Discussion

The aim of our study was to investigate whether oral administration of the NRTI stavudine produced hyperalgesia in rats and whether it also affected the overall condition of the rats, including gross motor function. Daily oral administration of stavudine at a dose of 50 $mg.kg^{-1}$ resulted in mechanical hyperalgesia in our rats within three weeks, and this hyperalgesia persisted throughout the six weeks of the study. Rats that received stavudine were more sensitive to the pressure applied to the tail by the bar algometer, such that the withdrawal latency to the noxious mechanical challenge, compared to the latency before stavudine administration, was decreased by 40 % to 50 % on average after three weeks of daily stavudine administration. While daily stavudine administration resulted in mechanical hyperalgesia from week three onward, hyperalgesia to the thermal challenge (49 ºC water) did not develop.

Although daily oral stavudine administration resulted in mechanical hyperalgesia within three weeks, other physiological functions which we measured, related to the overall condition of the rats, were not affected by the drug. Rats receiving stavudine gained weight normally, and consumed the same amount of food, per 100 g body mass, as rats receiving placebo. Voluntary wheel running activity also did not change with daily stavudine administration. Maximum running speed remained the same in rats given stavudine and placebo throughout the study, and both groups of rats showed similar increases in running distance over the first four weeks of the

study, followed by a decline in running distance in week five and week six, as they aged.

We stopped measuring for hyperalgesia in week six of the experiment, when we stopped administering the drug, but the pain induced by stavudine in humans typically resolves once drug administration ends [4]. Therefore it would be interesting to investigate whether the hyperalgesia in our rats also resolves after drug administration is stopped. In addition, even though we found no difference in the body mass of rats receiving stavudine or placebo, it is possible that the drug caused changes in body composition, as stavudine has been associated with lipodystrophy [22].

HIV-positive patients frequently experience spontaneous pain, such as lower limb pain and headache, making it difficult to perform normal daily activities and diminishing their quality of life [15,24]. Our finding that daily oral stavudine administration induced mechanical hyperalgesia without affecting appetite or voluntary activity suggests that stavudine did not cause spontaneous pain or allodynia (pain evoked by non-noxious challenges) to develop in our rats. Or, if spontaneous pain or allodynia did develop, the intensity of the pain was not sufficient to affect the normal growth and activity of the rats.

Although the oral bioavailabilty of stavudine is complete in rats, with 100 % of the dose reaching the systemic circulation [14], we found that mechanical hyperalgesia

to the bar algometer developed in the rat tail only after three weeks of daily oral stavudine administration, while Joseph and colleagues [11] showed that daily oral administration of 50 $mg.kg^{-1}$ of zalcitabine resulted in hyperalgesia in the rat hind paw within just seven days, as tested using von Frey filaments. The rate of onset of hyperalgesia may be different in the two anatomical sites or the techniques used to test for hyperalgesia may account for the differences in results. Joseph et al. [11] used a dynamic, punctuate stimulus, while we used a tonic, blunt stimulus. These two challenges may activate different nociceptors, which may explain why we observed mechanical hyperalgesia far later than Joseph and colleagues [11]. Also, differences in toxicity between the drugs may be responsible for the disparity between the two studies. Zalcitabine has a higher toxicity than stavudine, having the highest reported incidence of neuropathy in patients of all the NRTIs [6] and, in cell cultures, unlike stavudine, zalcitabine causes mitochondrial DNA depletion and decreases cell proliferation [5]. A further difference between our study and that of Joseph et al. [11] is that oral zalcitabine administration resulted in both mechanical and thermal hyperalgesia of the rat hind paw, whereas, in our study, stavudine induced only mechanical hyperalgesia in the rat tail. This difference also may result from the increased toxicity of zalcitabine compared to stavudine. Alternatively, the difference may be caused by differences in the thermal challenges employed. Joseph and colleagues [11] focused a radiant heat source on the rat paw, while we submerged the whole tail in 49 °C water. It may be possible that different nociceptive pathways are responsible for transmitting the thermal and mechanical challenges we employed, such that neurons responding to the noxious mechanical

challenge became sensitized following stavudine administration, while neurons responding to the noxious thermal challenge (49 °C water) did not [8].

Joseph et al. [11] also found that the paw withdrawal threshold of rats was decreased significantly for twenty days following a single intravenous injection of stavudine, from day one (50 $mg.kg^{-1}$) or day three (25 $mg.kg^{-1}$ and 10 $mg.kg^{-1}$) after injection, while daily oral administration of 50 $mg.kg^{-1}$ zalcitabine resulted in hyperalgesia in the hind paw after seven days [11]. Also, a single intraperitoneal injection of 25 $mg.kg^{-1}$ zalcitabine resulted in a decrease in the paw withdrawal threshold of rats for 42 days from day three after injection [2]. These results, along with the data presented here, show that the route of administration is a determinant of the hyperalgesia caused by NRTIs. The drugs, however, are administered orally to HIV-positive patients, so the time course we observed is likely to be more relevant to the management of HIV-positive patients, as it usually takes six to eight weeks of daily drug administration for HIV-positive patients to experience symptoms of peripheral neuropathy [10]. Also, our rats developed hyperalgesia rapidly between week two and week three of drug administration. This rapid onset of hyperalgesia after the drugs have been administered for some time, fits with the characteristic delayed onset of antiretroviral drug-induced pain in human patients on antiretroviral therapy [4,6].

Despite the presence of mechanical hyperalgesia in our rats, voluntary running activity was not affected. Throughout the study, our rats ran in a pattern consistent

with that observed for running distances in both male [21] and female [1] rats. Running distance increased significantly over the first four weeks and then decreased again toward baseline values as the rats aged. Maximum running speed remained constant throughout the study. Thus, although stavudine altered afferent nociceptive pathways, efferent pathways involved in gross motor activity were unaffected. Near lethal doses of stavudine administered orally to rabbits once daily for 24 weeks also did not result in signs of peripheral neurological deficits [30] and Joseph et al. [11] showed that 50 $mg.kg^{-1}$ zalcitabine, administered once intravenously into the tail vein, resulted in hyperalgesia but did not significantly affect motor function or co-ordination of rats, as tested on a rotarod [11]. In contrast, Morse et al. [23] found that oral administration of zalcitabine resulted in a dose- and time-dependent decrease in open-field locomotor activity of rats. Oral administration of zidovudine (AZT), which has very low neurotoxicity [6], had no effect on locomotion [23]. Thus, different NRTIs may have different effects on the locomotion of rats, related to the toxicity of the specific drug. Also, the same NRTI may have different effects on different types of locomotive activity, although the voluntary wheel-running we measured probably is more akin to the open-field activity measured by Morse and colleagues [23] than to the forced activity on the rotarod employed by Joseph et al. [11].

Although antiretroviral drug regimens may have side effects including gastrointestinal disturbances, such as nausea and diarrhoea, these adverse effects often are transient, occurring only in the early stages of antiretroviral therapy [22].

Stavudine also is more frequently associated with peripheral neuropathy than gastrointestinal disturbances [22]. Accordingly, our animals developed hyperalgesia with stavudine administration, but continued to thrive. Body mass increased by over 50 % in both groups of rats and each rat consumed approximately the same absolute amount of food every day of the study. The decrease in food intake, per 100 g body mass, in both groups of rats presumably simply was a consequence of ageing [28], indicating that growth and appetite were not affected by stavudine administration. Warner and colleagues [30] also showed that daily oral administration of stavudine to rabbits for 24 weeks did not result in changes in body mass or food intake.

In conclusion, we have shown that daily oral administration of the antiretroviral drug stavudine, using a novel technique for administering the drugs, resulted in mechanical hyperalgesia in rats within three weeks, and that this hyperalgesia persisted throughout the six weeks of the study. To our knowledge, this study is the first to demonstrate that extended daily stavudine administration does not adversely affect the overall condition of the rats. Voluntary running activity, appetite and growth did not differ between rats receiving stavudine and placebo, suggesting that the drug does not cause spontaneous pain. Further studies are required to determine possible causes for the mechanical hyperalgesia we have observed.

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CHAPTER THREE

PAPER TWO: Weber J., Mitchell D., Veliotes D., Mitchell B. and Kamerman P.R. Hyperalgesia induced by oral stavudine administration to rats does not depend on spinal release of the pro-inflammatory cytokines interleukin-6 or cytokine-induced neutrophil chemo-attractant-1, nor on spinal neuronal apoptosis or necrosis.

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Hyperalgesia induced by oral stavudine administration to rats does not depend on spinal release of the pro-inflammatory cytokines interleukin-6 or cytokine-induced neutrophil chemo-attractant-1, nor on spinal neuronal apoptosis or necrosis

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Abstract

To investigate whether central changes underlie the hyperalgesia induced by the nucleoside reverse transcriptase inhibitor (NRTI) stavudine in rats, we examined neuronal death and inflammatory cytokine secretion in the spinal cord. Stavudine (50 $mg.kg^{-1}$) or placebo was administered orally to Sprague-Dawley rats once daily for three or six weeks. Rats' responses to a blunt noxious mechanical challenge applied to their tails were recorded before and at the end of stavudine or placebo administration. Spinal cords excised after three or six weeks of drug or placebo administration either were examined for neuronal necrosis and apoptosis, or for cytokine-induced neutrophil chemo-attractant (CINC)-1 and interleukin (IL)-6. Daily stavudine administration induced mechanical hyperalgesia within three weeks, but increased CINC-1 concentrations only by six weeks. Neither the concentration of IL-6, nor the number of spinal cord neurones or the number of spinal apoptotic nuclei was affected by stavudine administration. Therefore, although six weeks of daily stavudine administration resulted in an increase in CINC-1 concentration in the spinal cord, the development of stavudine-induced hyperalgesia did not depend on increases in spinal concentrations of CINC-1 and IL-6, nor on apoptosis or necrosis of spinal cord neurones.

1. Introduction

Nucleoside reverse transcriptase inhibitors (NRTIs) are an integral part of highly active antiretroviral therapy (HAART), particularly in developing countries. These drugs frequently cause peripheral neuropathy and pain in HIV-positive patients [1,2], often leading to discontinuation of therapy or drug switching [2]. Although the precise mechanisms of NRTI-induced neuropathy are poorly understood, NRTI-related mitochondrial toxicity is thought to mediate the nerve damage caused by these drugs [1]. While long-term administration of NRTIs in HIV-positive patients is associated with decreased peripheral nerve fibre density, caused by axonal degeneration and the loss of small unmyelinated nerve fibres [1,3,4], and a single intraperitoneal injection of 25 $mg.kg^{-1}$ of the NRTI zalcitabine (ddC) resulted in structural changes of neurones in the sciatic nerves of rats [5], the effects of NRTI administration on neurones of the central nervous system are not well understood. Wallace et al. (2007) recently showed that repeated systemic injection of zalcitabine to rats resulted in a modest increase in microglial and astrocyte activity in the dorsal horn, with only a limited effect on dorsal root ganglion phenotype. The role of spinal neuronal damage and central pro-inflammatory cytokines in NRTI-induced neuropathy remains unexplored.

In rodent models of neuropathic pain, increased nociceptive hypersensitivity after peripheral nerve injury [6] is associated with microglial activation [7,8], the release of central pro-inflammatory cytokines [9-12] and neuronal death in the dorsal horn [6]. The NRTIs zalcitabine, didanosine (ddI), stavudine (d4T) and zidovudine

(AZT) also induce apoptosis of isolated rat dorsal root neurones [13,14] and decrease the regeneration of isolated rat neurones, following neurite removal [15]. If this NRTI-induced apoptosis and neuronal degeneration is not limited to isolated rat dorsal root neurones, but also occurs in the spinal cord of HIV-positive patients, it may contribute to the activation of glial cells and the release of pro-inflammatory cytokines, resulting in pain [3].

The South African National Antiretroviral Treatment Guidelines [16] recommend that the NRTI stavudine be prescribed as part of first-line HAART regimens, yet stavudine frequently is associated with the development of neuropathy [17]. We previously have shown that daily oral administration of the NRTI stavudine at a dose of 50 $mg.kg^{-1}$ resulted in mechanical hyperalgesia in the rat tail within three weeks, without affecting appetite and voluntary activity [18]. We now have investigated possible mechanisms of the hyperalgesia caused by oral stavudine administration in rats. As stavudine is known to cause nerve damage in vitro [13-15], and neuropathic pain is associated with neuronal death in the dorsal horn [6], we examined whether oral administration of stavudine induces apoptosis or necrosis of spinal neurones in rats. Secondly, as pro-inflammatory cytokines in the spinal cord are involved in the development of neuropathic pain [9-12,19], and possibly in NRTI-induced pain [3], we investigated whether daily oral administration of stavudine causes the release of cytokine-induced neutrophil chemo-attractant (CINC)-1 and interleukin (IL)-6 in the spinal cord of rats. Chemokines are thought to be involved in NRTI-induced neuropathy [5,20] and intracerebroventricular

injection of CINC-1 decreases the mechanical nociceptive threshold of rats [21], while IL-6 is increased in the spinal cord in other rat models of neuropathic pain [10,11]. Thus, the chemokine CINC-1 and the cytokine IL-6 may play a role in hyperalgesia caused by stavudine administration.

2. Materials and Methods

2.1. Animals

Experiments were performed on female Sprague-Dawley rats with an initial body mass of 199.8 ± 2.1 g that were housed individually and had free access to standard rat chow and water. All procedures were approved by the Animal Ethics Screening Committee of the University of the Witwatersrand (clearance no. 2005/26/3 & 2005/89/3) and are in accordance with the International Association for the Study of Pain (IASP)'s guidelines for pain research in animals [22].

2.2. Drug administration

Stavudine (2',3'-didehydro-3'-deoxythimidine, d4T; Zerit, Bristol-Myers Squibb, Johannesburg, South Africa) was administered orally once daily, at a dose of 50 $mg.kg^{-1}$, as a suspension set in a flavoured gelatine cube. Gelatine cubes were made by adding 7 ml savoury bread spread (Bovril, Unilever, Johannesburg, South Africa), 20 g cane sugar and 12 g unflavoured gelatine powder (Davis Gelatine, Johannesburg, South Africa) to 100 ml warm water [23]. The solution was aliquoted into 3 ml moulds and allowed to set. Stavudine-containing gelatine cubes were made by adding powdered stavudine to each aliquot, and mixing thoroughly

before the gelatine set. Placebo gelatine cubes did not contain stavudine. One gelatine cube was placed in the cage of each rat every morning at 09:00. The rats ate the gelatine cubes enthusiastically, and without spillage; spillage was visible easily because the spread imparted a dense black colour to the cubes.

2.3. Nociceptive testing

We tested for mechanical hyperalgesia by recording the withdrawal latency to a noxious mechanical challenge applied to the tail, with the rats placed in clear plastic restrainers that restricted trunk movement but allowed free movement of the tail. The rats were familiarized with the restrainers for three hours a day for three consecutive days before measurements began. A bar algometer with a 1 mm diameter probe (Haldex AB, Halmstad, Sweden), was placed across the dorsal surface of the middle of the tail and a static force of 4 N was applied [24]. The time taken for the rat to withdraw its tail was recorded with a stopwatch. The test was repeated three times for each rat at slightly displaced sites, with at least one minute between each measurement, and the average of the three measurements was recorded as the withdrawal latency for each rat. The algometer was removed from the tail if the rat had not reacted after 30 s. All measurements were made by the same observer between 09:00 and 12:00 in the morning. The withdrawal latency was recorded only when the rat displayed a clear tail withdrawal from the noxious challenge or the rat tried to turn around in the restrainer to get at the noxious challenge being applied to the tail. Other nondescript end-points, such as the rat starting to fidget, were ignored.

2.4. Experimental protocol

We used two separate groups of rats. In the first group, we tested rats' nociceptive function in response to daily stavudine administration, and killed the rats after three or six weeks of stavudine administration to take spinal cord samples for measuring apoptosis and necrosis. In the second group, nociceptive testing was not performed. Rats also were killed after three or six weeks of stavudine or placebo administration, and spinal cord samples taken to determine the expression of CINC-1 and IL-6.

2.4.1. Stavudine-induced hyperalgesia and histopathology

We recorded rats' withdrawal latencies to the noxious mechanical challenge once daily for five days before the start of stavudine administration. During this period rats were given placebo gelatine cubes every day. On the sixth day, experimental rats received gelatine cubes containing 50 $mg.kg^{-1}$ stavudine, and continued to be fed stavudine cubes once daily for three (n=5) or six (n=5) weeks. Control rats continued to receive placebo gelatine cubes once daily for three (n=5) or six (n=5) weeks. Nociceptive testing was repeated on the day before spinal cord samples were collected. An additional five treatment-naïve rats, which received neither stavudine nor placebo gelatine cubes and which were age-matched to rats in the stavudine and control groups did not undergo nociceptive testing but also were killed, and their spinal cords examined.

For spinal cord excision, rats were deeply anaesthetised with 1 ml sodium pentobarbital i.p. (Euthapent, 200 $mg.ml^{-1}$; Kyron Laboratories (Pty) Ltd., South Africa), before being perfused transcardially with heparinised saline (100 ml), followed by 4 % paraformaldehyde in 0.1 M phosphate buffer (pH=7.4) [28]. The entire lumbar spinal cord (L1 – L5) was removed and post-fixed in 4 % paraformaldehyde in phosphate buffer for 1 h at room temperature. Samples then were embedded in paraffin wax.

2.4.2. Stavudine-induced changes in cytokine synthesis

To investigate the effect of stavudine administration on cytokine concentrations, we removed the lumbar spinal cord of rats given daily 50 $mg.kg^{-1}$ stavudine gelatine cubes for three (n=5) or six (n=5) weeks. Control rats received placebo gelatine cubes for three (n=5) or six (n=5) weeks. An additional five treatment-naïve rats, age-matched to rats in the stavudine and control groups, also were killed and had their lumbar spinal cords removed and analysed.

For sample collection, rats were anaesthetised in a chamber perfused with 2 % isofluorane (Safeline Pharmaceuticals, Johannesburg, South Africa) and killed by intracardiac injection of 1 ml sodium pentobarbital (Euthanase, Kyron, Johannesburg, South Africa). The lumbar spinal cord was removed, weighed, flash frozen in liquid nitrogen and stored at -70 °C. For cytokine analysis, spinal cord samples were homogenised in 500 µl PBS (pH=7.4) containing 0.4 M NaCl, 0.05 % Tween-20, 0.5 % bovine serum albumin, 0.1 mM benzethonium chloride, 10 mM

EDTA and 20 $Kl.ml^{-1}$ aprotinin. The homogenates were centrifuged at 12 000 g for 60 min at 4 °C. The supernatant was analysed for CINC-1 and IL-6.

2.5. Histopathology

2.5.1. Neuronal density

To allow us to determine the number of dorsal horn and anterior horn neurones in a cross section of the spinal cord of each rat, 5 μm spinal cord sections were stained with haematoxylin and eosin (H&E) and neuronal cells counted, as previously described [25]. Sections were divided through the centre and analysed by light microscopy by a pathologist (BM), blinded to the treatment of the rat. Cells anterior to the central line were included in the anterior horns and those posterior to the line were included in the posterior (dorsal) horns. A neurone-specific marker was not used, as neurones were easily identified morphologically by the pathologist by their large size and characteristics of the nuclei and the amount of cytoplasm. The total number of dorsal horn and anterior horn neuronal nuclei was counted in up to five serial cross sections for each cord, giving an average number of neurones for the spinal cord of each rat.

2.5.2. Apoptosis

To allow us to examine neuronal apoptosis, 5 μm spinal cord sections were stained using a commercially available modified Terminal Deoxynucleotidyl Transferase-Mediated dUTP Nick End-Labelling (TUNEL) stain kit (DeadEnd™ Colorimetric

TUNEL System, Promega Corporation, Madison, WI, USA), as previously described [26]. Briefly, tissue sections were deparaffinised in xylene, gradually rehydrated in ethanol and then equilibrated in 0.85 % NaCl, followed by phosphate buffered saline (PBS, pH=7.4). Thereafter, spinal cord sections were fixed in 4 % paraformaldehyde in PBS, permeabilised with Proteinase K, and re-fixed with 4 % paraformaldehyde in PBS. Biotinylated nucleotides then were incorporated at the 3'-OH fragmented DNA ends of apoptotic nuclei using the terminal Deoxynucleotidyl Transferase (rTDT) enzyme in a humidified 37 ºC incubator, for one hour. The reaction was terminated by immersion in standard saline citrate (SSC, pH=7.2). Endogenous peroxidase activity was blocked by 0.3 % peroxide, after which horseradish peroxidase-labeled streptavidin (Streptavidin HRP) was bound to the biotinylated nucleotides, and coloured with the chromagen diaminobenzidine (DAB) and the peroxidase substrate hydrogen peroxide. Slides were rinsed in water, gradually dehydrated in ethanol and then xylene, and sealed with glass coverslips for analysis.

A positive control was included with each set of slides stained in order to demonstrate the efficacy of the assay. The enzyme DNAse was added to positive control slides after the second fixation step in paraformaldehyde, resulting in DNA fragmentation and positive staining for the TUNEL assay. Negative control slides also were included in each set of slides stained, in order to assess non-specific binding of the biotinylated nucleotides in the absence of the rTDT enzyme.

Using light microscopy, the number and location of apoptotic nuclei was recorded for up to five serial sections of each spinal cord [26]. In order to decrease the number of false positive results, cells were deemed to have stained TUNEL-positive only if the nucleus stained dark brown while the cytoplasm remained pale, as assessed by visual comparison of positive and negative control slides. As we found very few TUNEL-positive nuclei per spinal cord section we did not further examine apoptosis in this study by staining for activated caspase-3.

2.6. Cytokine assays

We determined CINC-1 and IL-6 concentrations in the spinal cord using an enzyme-linked immunosorbent assay (ELISA, National Institute of Biological Standards and Control, UK), as previously described [27]. Briefly, microtitre plates were coated overnight with sheep anti-rat polyclonal antibody. Volumes of 100 µl standard recombinant rat cytokine or sample was added to each well and left overnight at 4 °C. Sheep anti-rat biotinylated polyclonal antibodies were added at a 1:2000 dilution, and the sample incubated at room temperature (~22 ºC) for one hour. Finally, 100 µl of streptavidin-polyHRP (1:10000 dilution, Euroimmun, Cape Town, South Africa) was added to each well, at room temperature. After 30 minutes the plates were washed and the colour reagent o-Phenylenediamine dihydrochloride (40 µg in 100 µl per well, Sigma-Aldrich, South Africa) added. The reaction was terminated with H_2SO_4 (1 M, 150 µl per well) and the optical density measured at 490 nm. CINC-1 and IL-6 cytokine concentrations were analysed in duplicate using the appropriate sheep anti-rat polyclonal and biotinylated antibodies for each

cytokine. The detection limit of each assay, which allowed for the dilution factor of the sample, differed between CINC-1 and IL-6 assays, and is reported with the results of the assay.

2.7. Data analysis

Nociceptive and cytokine data are expressed as mean ± SEM. The average of the withdrawal latencies measured on the last three days before stavudine or placebo administration began served as a reference value against which changes in withdrawal latency were compared. Changes in withdrawal latencies to the noxious mechanical challenge were assessed using two-way Analysis of Covariance (ANCOVA) with group and time as the main effects, and rat mass as covariate. A Newman-Keuls post-hoc test was used if any of the main effects or interaction were significant. ANCOVA was used because previous experience has shown us that as rats grow, and their tails become thicker and the skin more keratinised, their response to the noxious mechanical challenge changes. Cytokine concentrations were assessed using two-way Analysis of Variance (ANOVA), with group and time as the main effects, and Newman-Keuls post-hoc comparisons if any of the main effects or interaction were significant.

The number of apoptotic nuclei and the number of dorsal horn and anterior horn neurones per spinal cord section are expressed as median (full range). The number of apoptotic nuclei and the number of dorsal horn and anterior horn neurones per spinal cord section were compared over time for rats administered stavudine or

placebo gelatine cubes using the Kruskal-Wallis test, with treatment-naïve rats (as time = 0) included in the comparison. Comparisons were made between rats receiving placebo and stavudine cubes at three weeks and six weeks using the Mann-Whitney test. Bonferroni correction for multiple comparisons was used for all non-parametric tests.

3. Results

3.1. Noxious mechanical challenge

Before stavudine or placebo administration, the withdrawal latency to the 4 N mechanical challenge applied to the tail was 5.4 ± 0.8 s for rats scheduled to receive placebo gelatine cubes and 6.3 ± 1.1 s for rats that were to receive stavudine gelatine cubes (t-test: t=1.96, P=0.08). Compared to rats receiving placebo cubes, and compared to withdrawal latencies before stavudine gelatine cube administration, there was a significant decrease in the withdrawal latencies of rats given stavudine for three weeks and six weeks (two-way ANCOVA; group effect: $F_{1,7}$=27.59, P<0.01; time effect: $F_{1,1}$=3.62, P=0.31; interaction: $F_{1,7}$=1.43, P=0.27; Figure 1).

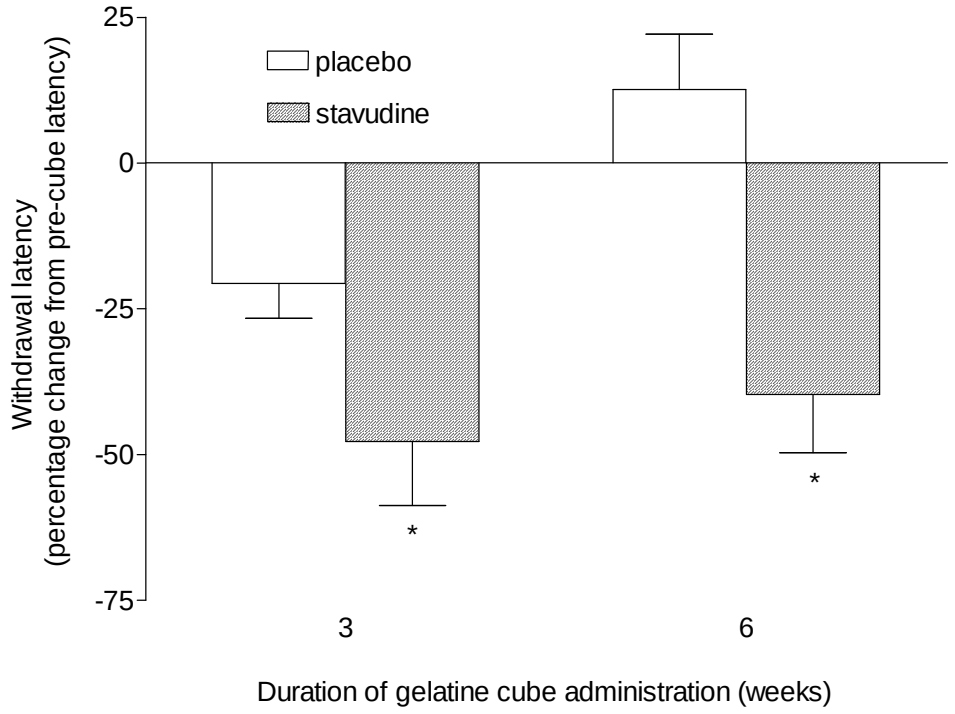

Figure 1. Changes in the withdrawal latencies (mean ± SEM) to a 4 N noxious mechanical challenge applied to the tail of rats given daily placebo (clear bars) or 50 $mg.kg^{-1}$ stavudine (hashed bars) orally in gelatine cubes. Withdrawal latencies are expressed as percentage change from latencies before cube administration. * indicates a significant difference in withdrawal latencies between the two treatment groups ($P<0.05$, ANCOVA with Newman-Keuls post-hoc comparisons)

3.2. Histopathology

3.2.1. Dorsal horn neuronal density

The number of dorsal horn neurones averaged on five serial sections of the lumbar spinal cord from each rat and then averaged across each group of rats receiving placebo or stavudine gelatine cubes is shown in Figure 2. The number of dorsal horn neurones did not differ over time for rats given placebo (Kruskal-Wallis Test; KW=2.90, P=0.94) or stavudine (Kruskal-Wallis Test; KW=0.20, P=3.69) or between rats receiving placebo or stavudine at three weeks (Mann-Whitney Test; U=9.50, P=2.19) or six weeks (Mann-Whitney Test; U=4.00, P=0.76).

3.2.2. Anterior horn neuronal density

The number of anterior horn neurones averaged on five serial sections of the lumbar spinal cord from each rat and then averaged across each group of rats receiving placebo or stavudine gelatine cubes also is shown in Figure 2. The number of anterior horn neurones did not differ over time for rats given placebo (Kruskal-Wallis Test; KW=8.73, P=0.05) or stavudine (Kruskal-Wallis Test; KW=7.61, P=0.09) or between rats receiving placebo or stavudine at three weeks (Mann-Whitney Test; U=1.00, P=0.06) or six weeks (Mann-Whitney Test; U=2.50, P=0.13).

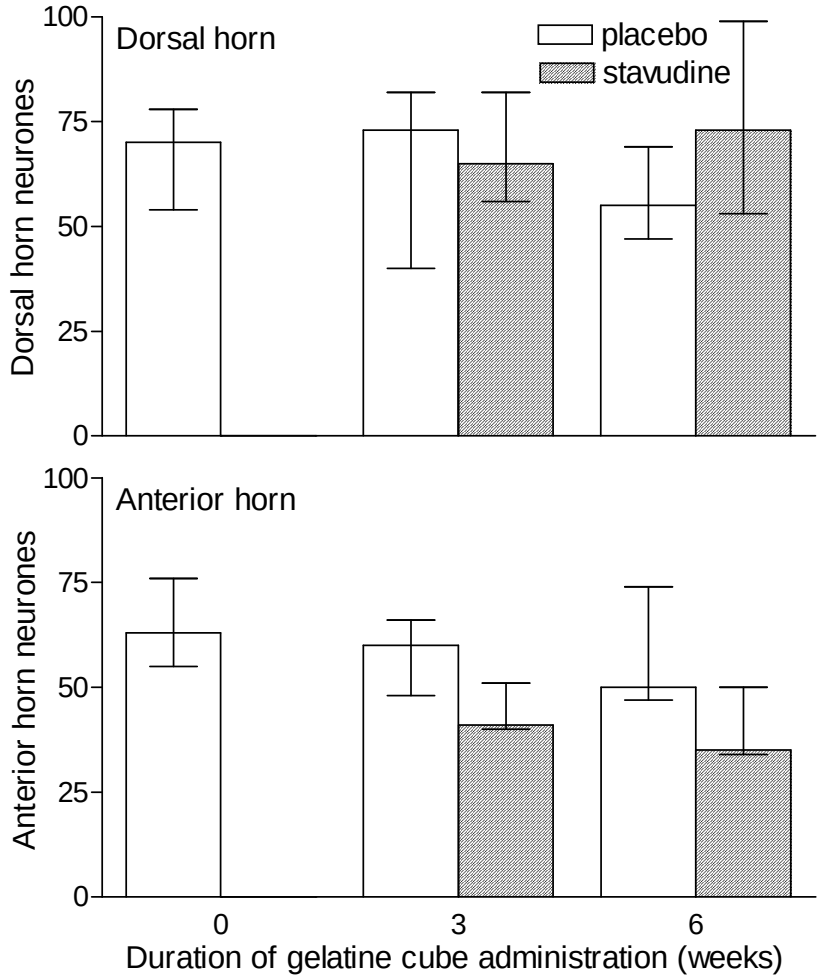

Figure 2. Dorsal horn and anterior horn neurones, median (full range), per spinal cord section of rats given placebo (clear bars) or 50 $mg.kg^{-1}$ stavudine (hashed bars) orally in gelatine cubes. Treatment-naïve rats are represented by week zero. There were no significant differences between treatment groups in the number of either dorsal horn or anterior horn neurones.

3.2.3. Apoptosis

As we found very few TUNEL-positive nuclei per spinal cord section, we did not determine the percentage of apoptotic nuclei per section. The absolute number of TUNEL-positive nuclei averaged on five serial sections of the lumbar spinal cord from each rat and then averaged across each group of rats receiving placebo or stavudine gelatine cubes is shown in Figure 3. The number of TUNEL-positive spinal cord nuclei did not differ over time for rats given placebo (Kruskal-Wallis Test; KW=0.82, P=2.66) or stavudine (Kruskal-Wallis Test; KW=0.82, P=2.66) or between rats receiving placebo or stavudine for three weeks (Mann-Whitney Test; U=8.00, P=2.92) or six weeks (Mann-Whitney Test; U=10.50, P=2.76).

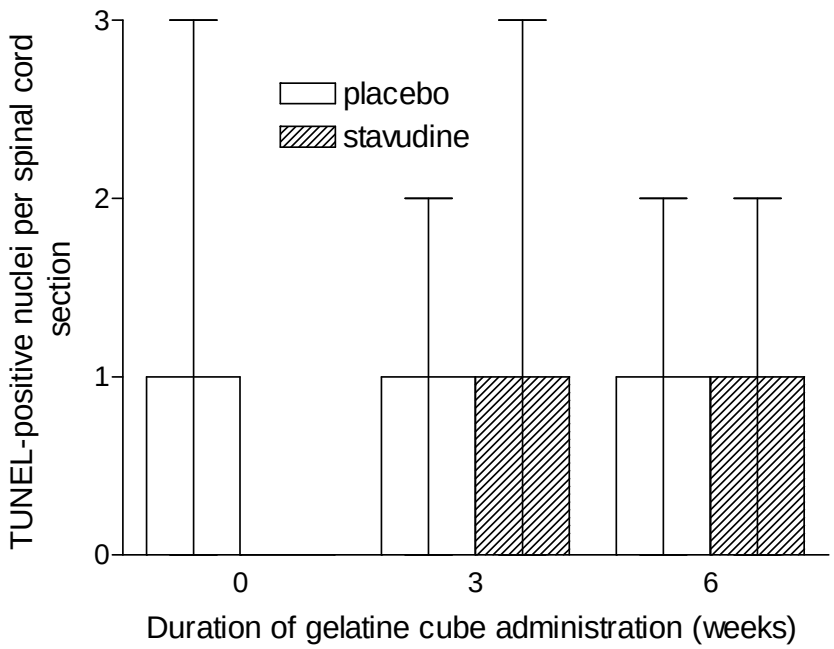

Figure 3. Apoptotic (TUNEL-positive) nuclei, median (full range), per spinal cord section of rats given placebo (clear bars) or 50 $mg.kg^{-1}$ stavudine (hashed bars) orally in gelatine cubes. Treatment-naïve rats are represented by week zero. There were no significant differences between treatment groups.

3.3. Spinal cord cytokine concentrations

Concentrations of CINC-1 and IL-6 in all spinal cord samples were above the detection limit of the assay.

3.3.1. Spinal cord CINC-1

Concentrations of CINC-1 in the spinal cord of rats receiving placebo or stavudine gelatine cubes are shown in Figure 4. Spinal cord CINC-1 concentrations were significantly elevated in rats receiving stavudine for six weeks, compared to all other groups of rats (two-way ANOVA; group effect: $F_{1,7}$=6.37, P=0.04; time effect: $F_{1,1}$=1.67, P=0.42; interaction: $F_{1,7}$=6.30, P=0.04).

3.3.2. Spinal cord IL-6

Concentrations of IL-6 in the spinal cord of rats receiving placebo or stavudine gelatine cubes also are shown in Figure 4. Spinal cord IL-6 concentrations did not differ between groups or with time (two-way ANOVA; group effect: $F_{1,7}$=0.03, P=0.87; time effect: $F_{1,1}$=1.03, P=0.49; interaction: $F_{1,7}$=4.23, P=0.08).

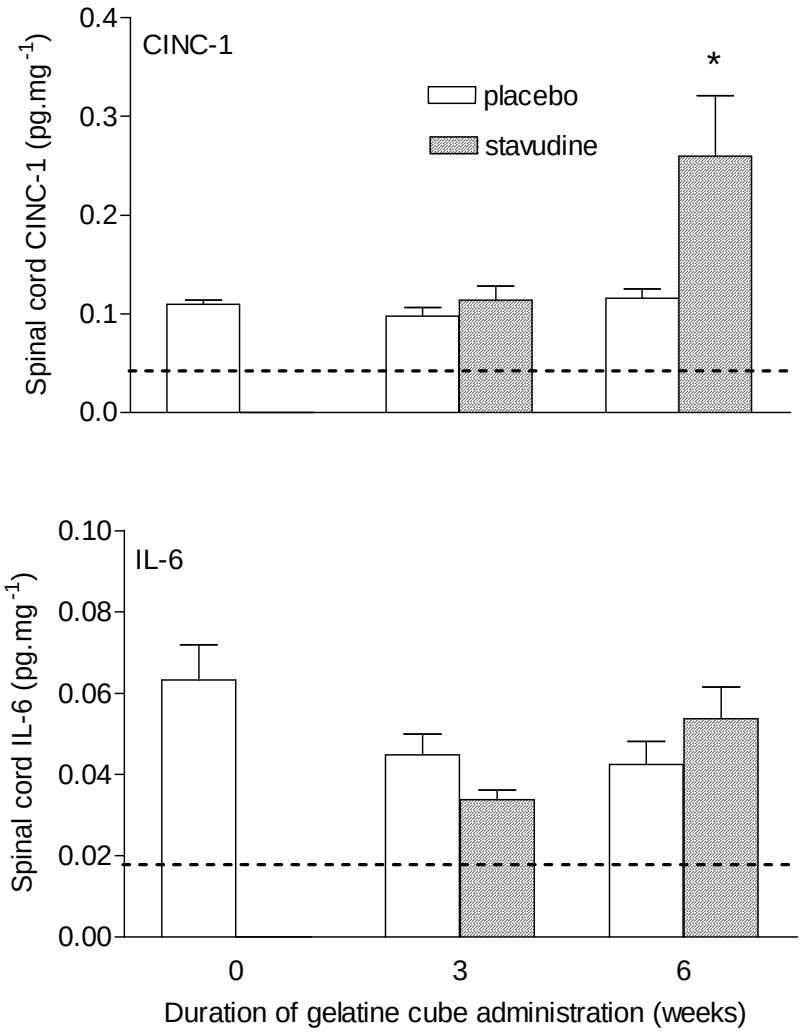

Figure 4. Spinal cord CINC-1 and IL-6 concentrations (mean ± SEM) of rats given placebo (clear bars) or 50 mg.kg^{-1} stavudine (hashed bars) orally in gelatine cubes. Treatment-naïve rats are represented by week zero. The dashed lines show the detection limits of the assays. * indicates a significant difference between rats receiving 50 mg.kg^{-1} stavudine daily for six weeks and all other groups of rats ($P<0.05$, ANOVA with Newman-Keuls post-hoc comparisons)

4. Discussion

We previously have shown that mechanical hyperalgesia develops in the tail of rats within three weeks of daily administration of 50 $mg.kg^{-1}$ stavudine [18]. The aim of our new study was to investigate possible mechanisms of this hyperalgesia by examining whether stavudine administration induces spinal nerve fibre death or causes the release of pro-inflammatory cytokines in the spinal cord. Daily oral administration of 50 $mg.kg^{-1}$ stavudine resulted in a pattern of mechanical hyperalgesia similar to that observed in our previous study [18]. Hyperalgesia was evident after the third week of drug administration, and still was present, at the same degree in week six, that is after three more weeks of stavudine administration. However, we found no evidence of stavudine-induced apoptosis or necrosis in the spinal cord, even following six weeks of daily stavudine intake. Neither the number of TUNEL-positive nuclei nor the number of dorsal horn or anterior horn neurones per spinal cord section was affected by stavudine administration. Spinal cord concentrations of IL-6 also remained unchanged following six weeks of daily stavudine administration. However, the concentration of CINC-1 in the lumbar spinal cord was elevated significantly in rats fed stavudine daily for six weeks, but not after three weeks of drug administration.

The elevation of CINC-1 well after hyperalgesia had developed indicates that CINC-1 may contribute to the long-term maintenance of stavudine-induced hyperalgesia, but the initial development of the hyperalgesia does not require increased spinal cord CINC-1. Neither the initiation nor the maintenance of

hyperalgesia requires increased spinal cord IL-6 and it still is unclear how the hyperalgesia first develops. It may be necessary to examine more closely changes in the spinal cord occurring after one or two weeks of daily stavudine administration, when hyperalgesia has not yet developed, to determine whether other central mechanisms are involved in the development of stavudine-induced hyperalgesia. In our study we only examined spinal concentrations of CINC-1 and IL-6, but other pro-inflammatory cytokines, including IL-1β [9,10,12] and tumour necrosis factor (TNF)-α [9,10], also are elevated in the spinal cord in other rodent models of peripheral neuropathy, and may be involved in stavudine-induced hyperalgesia. Also, with the elevation in CINC-1 in the spinal cord after six weeks of daily stavudine administration, it would be worthwhile, in a future experiment, to administer stavudine for longer, and to check for an increased incidence of cell death in later weeks.

Expression of CINC-1 increases following injury to the central nervous system [29,30], with CINC-1 production being more pronounced in the spinal cord than in the brain [30]. Once CINC-1 is expressed, it attracts neutrophils to the damaged region, which may exacerbate damage to the affected area, as these cells destroy tissue by releasing free radicals [4,31] and proteolytic enzymes [32], as well as by phagocytosis [33]. However, in our study, there was no evidence of spinal cord damage, and the hyperalgesia we observed was no more intense when CINC-1 was elevated after six weeks of daily stavudine administration than it was after three weeks, before CINC-1 was elevated. Moreover, there was no deterioration in

running activity [18], which is dependent on a functional spinal cord, when CINC-1 was increased. Thus, the elevation of CINC-1 in the spinal cord of rats, administered stavudine daily for six weeks, probably was not caused by nerve damage in the spinal cord. Wallace et al. (2007) recently showed that systemic administration of zalcitabine to rats resulted in moderate microgliosis and astrocytosis in the dorsal horn. Therefore, the increased concentration of CINC-1 we observed following stavudine administration also may have been the result of microglial and astrocyte activation [34] in response to peripheral nerve injury [35] induced by stavudine [1,3,4].

Though the concentration of the chemokine CINC-1 was increased following stavudine administration in our study, concentrations of IL-6 in the spinal cord were not elevated, although production of this cytokine also is increased in the spinal cord in other rat models of peripheral neuropathy [10,11]. IL-6 plays a role in the creation and continuation of other types of neuropathic pain [19], but this cytokine does not appear to be involved in the maintenance of stavudine-induced hyperalgesia, as it was not increased in the spinal cord after six weeks. While IL-6 also was not elevated in the spinal cord after three weeks, indicating that it probably is not involved in the development of stavudine-induced hyperalgesia, we cannot exclude the possibility that IL-6 was elevated before the onset of the hyperalgesia.

Whereas the hyperalgesia caused by other models of neuropathic pain is accompanied by vast changes in cytokine activity in the spinal cord [9-12], and

neuronal death in the dorsal horn [6], we have no evidence that the hyperalgesia induced by stavudine administration resulted from plasticity in the central nervous system. While stavudine induces apoptosis and neuronal degradation of isolated rat neurones [13-15], daily oral stavudine administration did not cause apoptosis or necrosis of spinal cord neurones in rats in our study. Similarly, Wallace et al (2007) found that systemic administration of zalcitabine to rats caused only minimal changes in dorsal root ganglion activity, although paw withdrawal threshold was decreased. As stavudine administration is associated with loss of peripheral neurones in HIV-positive patients [1,3,4], and repeated injections of zalcitabine to rats resulted in decreased intra-epidermal nerve fibre density [20], stavudine administration to rats also may have caused damage to peripheral neurones. Damage to peripheral neurones initiates the release of excitatory amino acids, prostaglandins and nitric oxide from activated glial cells [19] and may result in up- or down-regulation of genes responsible for the excitability of the neurones [36], inducing a heightened sensitivity to nociceptive input [19,36]. Similar changes, caused by injury to peripheral neurones, may account for the mechanical hyperalgesia we observed following daily oral administration of stavudine to rats.

In conclusion, we have shown that hyperalgesia that develops with chronic daily oral administration of the NRTI stavudine to rats does not involve apoptosis or necrosis of spinal neurones, or elevation of spinal secretion of the inflammatory cytokine IL-6. Few studies have examined concentrations of both IL-6 and CINC-1 in the spinal cord following central or peripheral nerve injury, but we have shown

that daily oral administration of stavudine increases the production of CINC-1, while the concentration of IL-6 remains unaffected. Stavudine administration resulted in an increase in the concentration of CINC-1 in the spinal cord, as assessed after six weeks of stavudine administration, up to three weeks after the rats developed hyperalgesia, which indicates that CINC-1 is not essential for initiating the hyperalgesia. Further studies are required to examine other mechanisms, both centrally and peripherally, which may be responsible for the hyperalgesia we observed with chronic stavudine administration to rats.

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CHAPTER FOUR

PAPER THREE: Weber J., Mitchell D. and Kamerman P.R. Oral administration of stavudine to rats at a dose inducing hyperalgesia does not affect plasma lactate, adiponectin, cytokine-induced neutrophil chemo-attractant-1 or interleukin-6 concentrations.

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Oral administration of stavudine to rats at a dose inducing hyperalgesia does not affect plasma lactate, adiponectin, cytokine-induced neutrophil chemo-attractant-1 or interleukin-6 concentrations

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Short title: Stavudine and plasma lactate and cytokines

Keywords: antiretroviral therapy; nucleoside reverse transcriptase inhibitor (NRTI); 2',3'-didehydro-3'-deoxythimidine (d4T); chemokines; cytokines; lipodystrophy; mitochondrial toxicity

Abstract

Stavudine, a nucleoside reverse transcriptase inhibitor (NRTI) used to treat HIV infection, causes side effects in HIV-positive patients, including mitochondrial toxicity, lipodystrophy and peripheral neuropathy. These conditions are associated with increased plasma lactate, decreased plasma adiponectin and increased plasma pro-inflammatory cytokine concentrations. It is not yet clear whether stavudine is intrinsically toxic, or whether its side effects are confined to patients compromised by HIV. To investigate stavudine-induced changes in mitochondrial bioenergetics, fat distribution, and circulating pro-inflammatory cytokine concentrations in rats exhibiting the neurological phenomenon of hyperalgesia, we administered stavudine (50 $mg.kg^{-1}$) orally to Sprague-Dawley rats once daily for three or six weeks, in gelatine cubes, and measured plasma lactate, adiponectin, cytokine-induced neutrophil chemo-attractant (CINC)-1 and interleukin (IL)-6. Control rats received cubes without stavudine. Plasma lactate, adiponectin, CINC-1 and IL-6 concentrations were unchanged in rats following three or six weeks of daily stavudine administration. We have shown that stavudine-related mitochondrial toxicity and fat redistribution, if present, were insufficient to significantly alter lactate and adiponectin production in rats, and that circulating CINC-1 and IL-6 are unlikely to be involved in the development or maintenance of the hyperalgesia induced by stavudine in rats. Stavudine toxicity appears to be exacerbated in HIV-positive patients.

1. Introduction

Stavudine (d4T) is a nucleoside reverse transcriptase inhibitor (NRTI) recommended by the South African National Antiretroviral Treatment Guidelines [1,2] as part of first-line highly-active antiretroviral therapy (HAART). NRTIs frequently cause peripheral neuropathy and pain in HIV-positive patients [3,4], and also are associated, in HIV-positive patients, with other adverse effects, such as lipodystrophy and lactic acidosis [5]. Many of these side effects are related to NRTI-induced mitochondrial toxicity, which results in delayed cell doubling and decreased mitochondrial DNA content [3,6,7], possibly by inhibition of DNA polymerase-γ activity [6]. Mitochondrial toxicity causes alterations in mitochondrial bioenergetics [6], increasing anaerobic respiration and lactate buildup [7-9]. Thus plasma lactate concentration frequently is increased in HIV-positive patients on stavudine-containing therapy [10-12].

Stavudine also has been implicated in the development of lipodystrophy, the fat redistribution characterised by peripheral fat loss and central fat accumulation, in HIV-positive patients [13]. Lindegaard et al. (2004) found that 90 % of patients who had previously received or currently were receiving stavudine developed lipodystrophy. Lipodystrophy [14,15] and stavudine use [15,16] are associated with decreased plasma levels of adiponectin, an adipocytokine produced and secreted by adipose tissue, which may play a role in glucose metabolism [17]. Lindegaard and colleagues (2004) found that lipodystrophy further was associated with increased plasma levels of the pro-inflammatory cytokines interleukin (IL)-6, and tumour

necrosis factor (TNF)-α, in HIV-positive patients receiving stavudine [15]. Circulating pro-inflammatory cytokines are thought to play a role in neuropathic pain [18], such as may occur in HIV-positive patients treated with antiretroviral medication [3,4].

Though stavudine clearly produces toxic side effects in HIV-positive patients, what is less clear is whether stavudine is intrinsically toxic, or is toxic only in patients already compromised by HIV infection. A recent study showed that a paediatric dose (10 mg) of stavudine administered to healthy volunteers for eight days had no adverse effects on the general wellbeing of the volunteers [19]. While eight days may have been insufficient to induce symptoms of neuropathy in healthy volunteers, stavudine has been shown to cause hyperalgesia after one day [20] and changes in fat mass within two weeks [21,22] in otherwise-healthy rodents, indicating that stavudine indeed appears to be intrinsically toxic. We have found previously that daily oral administration of stavudine to rats resulted in mechanical hyperalgesia of the tail within three weeks [23] and that this hyperalgesia is not dependent on inflammatory changes in the spinal cord (unpublished results), a recognised cause of hyperalgesia in other circumstances [24-28]. We now have investigated whether prolonged oral administration of stavudine induces changes in plasma concentrations of lactate, adiponectin and the cytokines, CINC-1 and IL-6. We examined plasma levels after three weeks, when hyperalgesia first was evident in our previous study and after six weeks, when hyperalgesia was well established [23].

2. Materials and Methods

2.1. Animals

Experiments were performed on female Sprague-Dawley rats with an initial mass of 199.8 ± 10.8 g, which were housed individually and had free access to standard rat chow and water. All procedures were approved by the Animal Ethics Screening Committee of the University of the Witwatersrand (clearance no. 2005/26/3).

2.2. Drug administration

Stavudine (2',3'-didehydro-3'-deoxythimidine, d4T; Zerit, Bristol-Myers Squibb, Johannesburg, South Africa) was administered orally once daily, at a dose of 50 $mg.kg^{-1}$, as a suspension set in a flavoured gelatine cube. Gelatine cubes were made by adding 7 ml savoury bread spread (Bovril, Unilever, Johannesburg, South Africa), 20 g cane sugar and 12 g unflavoured gelatine powder (Davis Gelatine, Johannesburg, South Africa) to 100 ml warm water [29]. The solution was allowed to set in 3 ml moulds. Stavudine-containing gelatine cubes were made by adding powdered stavudine to each aliquot, and mixing thoroughly before the gelatine set. Placebo gelatine cubes were identical but did not contain stavudine. One gelatine cube was placed in the cage of each rat every morning at 09:00. The rats ate the gelatine cubes enthusiastically, and without spillage; any spillage would have been visible easily because the spread imparted a dense black colour to the cubes.

2.3. Experimental protocol

We took blood samples from rats given stavudine or placebo gelatine cubes daily for three (n=5) or six (n=5) weeks. An additional five treatment-naïve rats, age-matched to rats in the stavudine and control groups, also were killed, and had blood samples taken and analysed. For blood collection, rats were anaesthetised in a chamber perfused with 2 % isofluorane (Safeline Pharmaceuticals, Johannesburg, South Africa). Blood was collected by cardiac puncture into sterile tubes containing EDTA and the rats then were killed by intracardiac injection of 1 ml sodium pentobarbital (Euthanase, Kyron, Johannesburg, South Africa). Blood samples were centrifuged at 2000 g for 15 minutes at 4 °C. The plasma was removed and stored at -70 °C until assayed.

2.4. Assays

We determined lactate concentrations in the plasma using a commercially-available lactate assay kit (EnzyChrom^{TM}, BioAssay Systems, California, USA), following the manufacturer's instructions. Adiponectin concentrations were determined using a commercially-available rat adiponectin enzyme-linked immunosorbent assay (ELISA) kit (LINCO Research, Missouri, USA), following the manufacturer's instructions.

CINC-1 and IL-6 concentrations in the plasma also were determined using an ELISA (National Institute of Biological Standards and Control, UK), as described previously [30]. Briefly, microtitre plates were coated overnight with sheep anti-rat

polyclonal antibody. Volumes of 100 µl standard recombinant rat cytokine or plasma sample were added to each well and left overnight at 4 °C. Sheep anti-rat biotinylated polyclonal antibodies were added at a 1:2000 dilution, and the sample incubated at room temperature (~22 ºC) for one hour. Finally, 100 µl of streptavidin-polyHRP (1:10000 dilution, Euroimmun, Cape Town, South Africa) was added to each well, at room temperature. After 30 minutes the plates were washed and the colour reagent o-Phenylenediamine dihydrochloride (40 µg in 100 µl per well, Sigma-Aldrich, South Africa) added. The reaction was terminated with H_2SO_4 (1 M, 150 µl per well) and the optical density measured at 490 nm.

Lactate, adiponectin, CINC-1 and IL-6 concentrations in each sample were analysed in duplicate. The detection limit of each assay, which allowed for the dilution factor of the sample, differed between lactate, adiponectin, CINC-1 and IL-6 assays, and is reported with the results of the assays.

2.5. Data analysis

Data are expressed as mean ± SEM. Lactate, adiponectin and cytokine concentrations were assessed using two-way Analysis of Variance (ANOVA), with group and time as the main effects. Newman-Keuls post-hoc comparisons were used if any of the main effects or interaction were significant.

3. Results

3.1. Plasma lactate concentrations

Concentrations of lactate in all plasma samples were above the detection limit of the assay. Plasma lactate concentrations of rats receiving placebo or stavudine gelatine cubes and treatment-naïve rats are shown in Figure 1. Plasma lactate concentrations did not differ between groups or with time (two-way ANOVA; group effect: $F_{1,8}$=1.30, P=0.29; time effect: $F_{1,1}$=7.16, P=0.23; interaction: $F_{1,8}$=2.38, P=0.16).

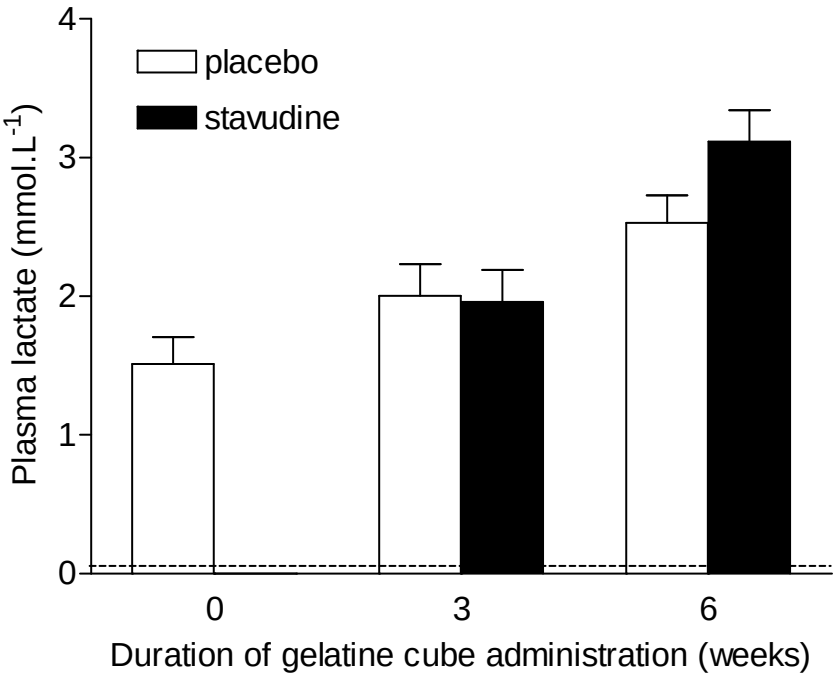

Figure 1. Plasma lactate concentrations (mean ± SEM) of rats given placebo (clear bars) or 50 $mg.kg^{-1}$ stavudine (solid bars) orally in gelatine cubes. Treatment-naïve rats are represented by week zero. The dashed line shows the detection limit of the assay. There were no significant differences between any groups.

3.2. Plasma adiponectin concentrations

Concentrations of adiponectin in all plasma samples were above the detection limit of the assay. Plasma adiponectin concentrations of rats receiving placebo or stavudine gelatine cubes and treatment-naïve rats are shown in Figure 2. Plasma adiponectin concentrations did not differ between groups or with time (two-way ANOVA; group effect: $F_{1,8}$=1.83, P=0.21; time effect: $F_{1,1}$=0.23, P=0.72; interaction: $F_{1,8}$=0.24, P=0.64).

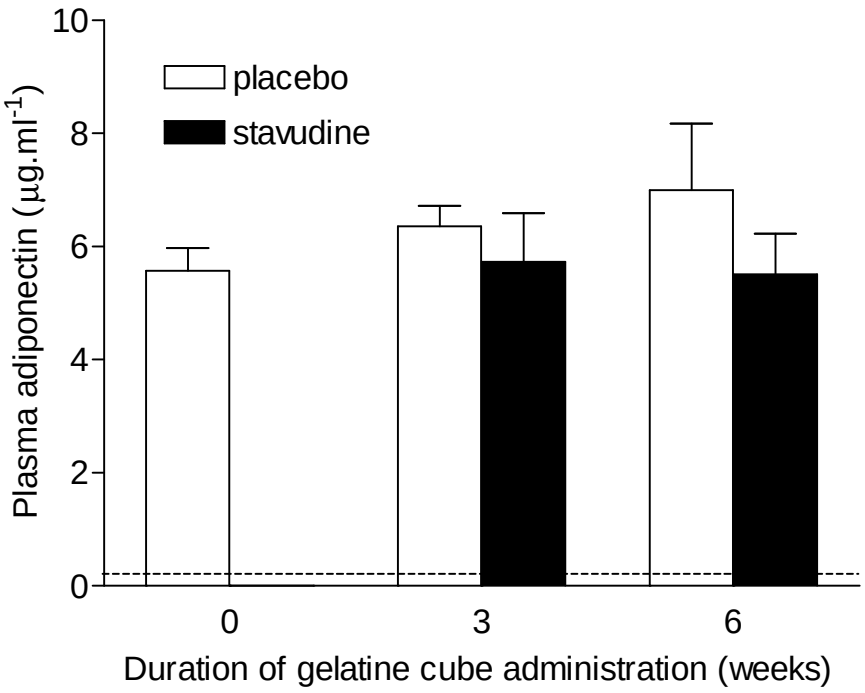

Figure 2. Plasma adiponectin concentrations (mean ± SEM) of rats given placebo (clear bars) or 50 $mg.kg^{-1}$ stavudine (solid bars) orally in gelatine cubes. Treatment-naïve rats are represented by week zero. The dashed line shows the detection limit of the assay. There were no significant differences between any groups.

3.3. Plasma pro-inflammatory cytokine concentrations

Concentrations of CINC-1 and IL-6 in all plasma samples were above the detection limit of the assays.

3.3.1. Plasma CINC-1

Plasma CINC-1 concentrations of rats receiving placebo or stavudine gelatine cubes and treatment-naïve rats are shown in Figure 3. Plasma CINC-1 concentrations did not differ between groups or with time (two-way ANOVA; group effect: $F_{1,8}$=1.68, P=0.23; time effect: $F_{1,1}$=0.37, P=0.65; interaction: $F_{1,8}$=2.21, P=0.18).

3.3.2. Plasma IL-6

Plasma IL-6 concentrations of rats receiving placebo or stavudine gelatine cubes and treatment-naïve rats also are shown in Figure 3. Plasma IL-6 concentrations did not differ between groups or with time (two-way ANOVA; group effect: $F_{1,8}$=0.64, P=0.45; time effect: $F_{1,1}$=2.81, P=0.34; interaction: $F_{1,8}$=1.92, P=0.20).

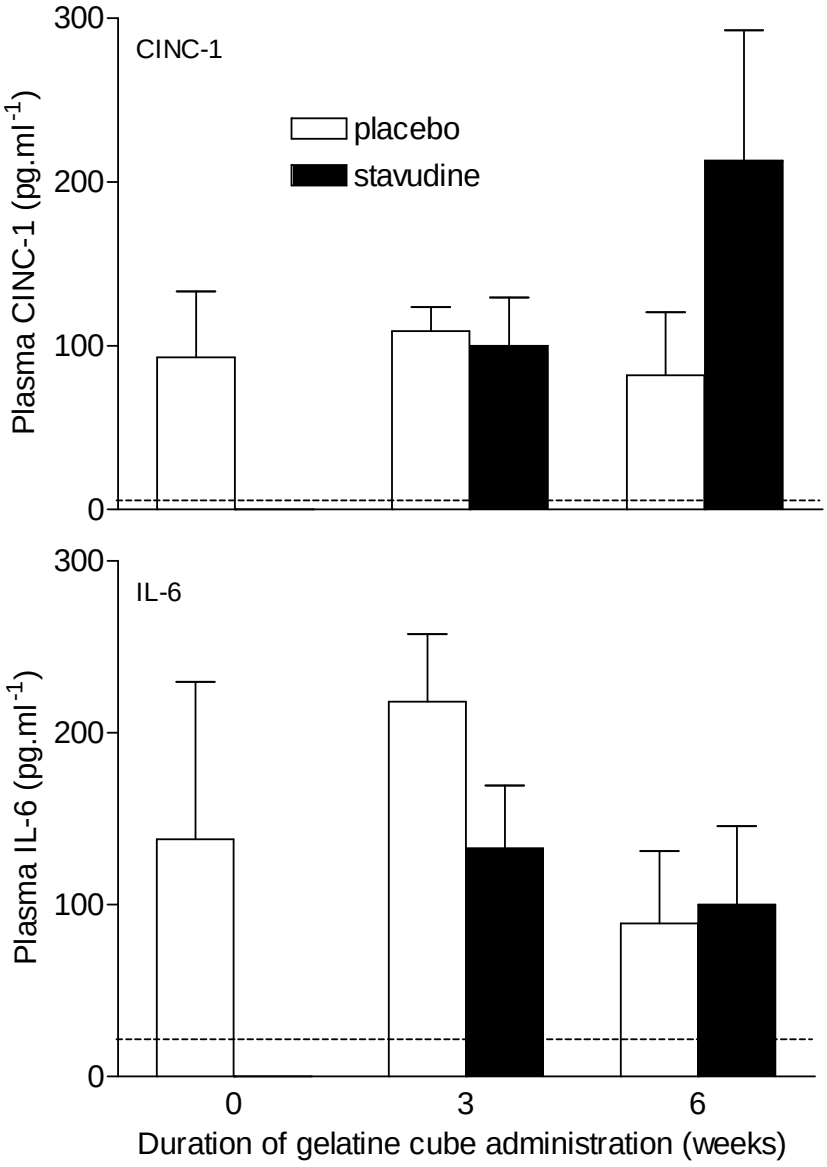

Figure 3. Plasma CINC-1 and IL-6 concentrations (mean ± SEM) of rats given placebo (clear bars) or 50 $mg.kg^{-1}$ stavudine (solid bars) orally in gelatine cubes. Treatment-naïve rats are represented by week zero. The dashed lines show the detection limits of the assays. There were no significant differences between any groups, for either cytokine.

4. Discussion

We have shown previously that daily oral administration of the NRTI stavudine to rats at a dose of 50 $mg.kg^{-1}$ resulted in mechanical hyperalgesia within three weeks, without affecting the overall condition of the rats [23]. We have shown now that the neurological changes responsible for the hyperalgesia occurred without physiological changes in mitochondrial activity, as reflected in plasma concentrations of lactate, and that stavudine administration was not associated with changes in plasma concentrations of adiponectin. We also have shown that the neurological changes are not accompanied by changes in plasma concentrations of two pro-inflammatory cytokines, CINC-1 and IL-6. We have shown that plasma concentrations of lactate, adiponectin, CINC-1 and IL-6 did not change significantly over the period of stavudine administration, and did not differ significantly, at any time, from the concentrations in rats receiving placebo.

We previously have found that the concentration of CINC-1 is increased in the lumbar spinal cord of rats receiving stavudine once daily for six weeks (unpublished results) but in this study we found that the concentration of CINC-1 in the plasma did not differ between groups. It is possible that there was an increase in the plasma concentration of CINC-1 in rats receiving oral stavudine daily for six weeks but that we were unable to detect this difference because the sample size was too small. Given the similarity in concentrations between those of rats receiving stavudine and those receiving placebo, it is highly unlikely that the absence of a rise in lactate and adiponectin concentrations was the result of statistical error. It is

possible, nevertheless, that stavudine indeed induced mitochondrial toxicity and fat redistribution, but that these metabolic changes did not result in an increased lactate and a decreased adiponectin concentration in the plasma, as observed in HIV-positive patients on stavudine-containing therapy [10-12]. In a future study, it would be advisable to examine other indices of mitochondrial toxicity, such as mitochondrial DNA content [31,32], and to determine the actual body fat distribution of rats given stavudine, to further examine the possibility of mitochondrial toxicity and lipodystrophy in otherwise-healthy rats given stavudine.

In a previous study we found that daily oral administration of stavudine did not affect growth, appetite or voluntary activity in rats, despite inducing mechanical hyperalgesia within three weeks [23]. We now have failed to find evidence that stavudine significantly altered mitochondrial bioenergetics in rats, although administration of other NRTIs has been shown to adversely affect mitochondrial function in rats [32,33] . While lactic acidosis is a well-recognised adverse event in HIV-positive patients taking stavudine [10-12] and stavudine also is associated with decreased mitochondrial function in cell culture [6,34], we found that daily stavudine administration for three or six weeks did not significantly elevate plasma lactate concentrations in rats. Our findings are in agreement with those of Note et al. (2003), who showed that plasma lactate levels were unchanged in mice receiving a high dose of stavudine daily for two weeks [35], and with the findings of Lewis and colleagues (2005) who found that plasma lactate levels were not elevated in mice receiving antiretroviral drug combination therapy containing stavudine for 35

days [36]. In another study, mice receiving a very high dose of stavudine (500 $mg.kg^{-1}$) once daily for two weeks also did not show an increase in plasma lactate concentration although stavudine did induce fat wasting [21]. Although administration of other NRTIs to rats is associated with a decrease in mitochondrial DNA [32,33] and alterations in the functioning of the mitochondrial electron transport chain [32,33], the toxicity of NRTIs varies [4] and the effect of stavudine on mitochondrial function in otherwise-healthy rodents remains unclear. The results of our and other studies examining the effect of stavudine on plasma lactate concentrations indicate that mitochondrial toxicity induced by stavudine in otherwise-healthy rodents appears to be inadequate to cause an increase in anaerobic respiration great enough to result in significant lactate buildup.

Besides highlighting the limited effect of stavudine on mitochondrial function in rats, we also have shown that daily oral administration of stavudine to rats did not significantly affect fat distribution, at least as reflected in plasma adiponectin concentration. Our results correspond to those by Maisonneuve et al. (2004), who showed that, although stavudine administration did alter body fat mass in mice, plasma adiponectin concentration remained unchanged throughout the study. These and our findings imply that stavudine administration on its own does not result in sufficient changes in fat distribution to cause the decreased plasma adiponectin concentration observed in HIV-positive patients with stavudine-induced lipodystrophy [15].

We previously have shown that, in rats, there is no clear increase in spinal pro-inflammatory cytokines accompanying stavudine-induced mechanical hyperalgesia, with only the concentration of CINC-1 being elevated in the spinal cord of rats administered stavudine daily for six weeks (unpublished results). Our finding that plasma concentrations of CINC-1 and IL-6 were not altered by daily administration of 50 $mg.kg^{-1}$ stavudine indicates that these circulating pro-inflammatory cytokines, which are elevated in the plasma of rats in other models of hyperalgesia [37,38], also are unlikely to be involved in the development or maintenance of stavudine-induced mechanical hyperalgesia [23], although we cannot exclude the possibility that these cytokines were elevated in the plasma before the onset of the hyperalgesia. Instead, as stavudine administration is associated with loss of peripheral neurones in HIV-positive patients [3,39,40], and repeated injections of zalcitabine to rats resulted in decreased intra-epidermal nerve fibre density [41], stavudine administration to rats also may have caused damage to peripheral neurones. Damage to peripheral neurones initiates the release of excitatory amino acids, prostaglandins and nitric oxide from activated glial cells [28] and may result in up- or down-regulation of genes responsible for the excitability of the neurones [42], inducing a heightened sensitivity to nociceptive input [28,42]. Similar changes, rather than pro-inflammatory cytokine release, may account for the mechanical hyperalgesia we observed in rats following daily oral administration of stavudine [23].

In contrast to the lack of change of plasma concentrations in rats given stavudine, plasma concentrations of lactate [10-12] and pro-inflammatory cytokines [15] frequently are increased, while adiponectin concentrations often are decreased [15,16], in HIV-positive patients on stavudine-containing therapy. Our results and the contrasting results in HIV-positive patients are consistent with the suggestion that HIV infection is necessary for toxic neuropathy, the nerve damage induced by antiretroviral drugs, to develop [41,43]. It is possible that, just as antiretroviral drugs, particularly NRTIs, are thought to exacerbate the nerve damage initially caused by the virus [41,43], HIV infection also is necessary for other adverse effects of NRTI therapy to arise. The underlying HIV infection and accompanying immune suppression may enhance the side effects of NRTI therapy, such that these adverse effects are more pronounced in HIV-positive patients than in animals administered NRTIs in the absence of HIV infection.

Although stavudine administration to rats results in hyperalgesia [20,23] and NRTIs adversely affect mitochondrial function at a cellular level in rats, decreasing mitochondrial DNA [32], altering mitochondrial morphology [8,44] and affecting the functioning of the mitochondrial electron transport chain [33], these changes do not appear to affect the overall condition of the rats [23]. In contrast, the numerous side effects associated with antiretroviral therapy in HIV-positive patients frequently diminish quality of life in these patients, often resulting in anxiety and depression [45-47], indicating that the toxicity of NRTIs probably is exacerbated by

HIV infection, thereby increasing the severity of NRTI-induced adverse events in immuno-compromised individuals.

In conclusion, we have found that daily oral administration of 50 $mg.kg^{-1}$ stavudine to rats for three or six weeks did not alter plasma concentrations of lactate, adiponectin, CINC-1 or IL-6. These findings indicate that stavudine has limited effects on mitochondrial bioenergetics and fat distribution, at least as reflected in plasma adiponectin concentration, and that circulating levels of the pro-inflammatory cytokines CINC-1 and IL-6 are unlikely to be involved in the development or maintenance of stavudine-induced hyperalgesia, in otherwise-healthy rats.

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CHAPTER FIVE

CONCLUSION

The NRTI stavudine, which is used to treat HIV infection, is associated with peripheral neuropathy and pain in HIV-positive patients (Dalakas, 2001; Moyle & Sadler, 1998; Simpson & Tagliati, 1995) and causes pain hypersensitivity in otherwise-healthy rats (Joseph et al., 2004). The mechanisms of NRTI-induced toxic neuropathy and pain are not well understood, partly because of a lack of robust animal models of the disease process. In particular, few studies have examined the effects of stavudine, a frequently prescribed antiretroviral drug in South Africa (Grimwood, 2004), on pain sensitivity in animals, as most researchers investigate the more toxic, yet seldom prescribed NRTI zalcitabine instead.

Thus, the primary aim of my PhD was to investigate how chronic daily oral administration of the NRTI stavudine affects nociception in rats, and specifically, whether stavudine induces hyperalgesia. In my first study I showed that daily oral administration of 50 $mg.kg^{-1}$ stavudine, using a novel technique for administering the drug, resulted in mechanical hyperalgesia in rats within three weeks, and that this hyperalgesia persisted throughout the six weeks of the study. In contrast, Joseph and colleagues (2004) showed that chronic daily oral administration of the NRTI zalcitabine to rats induced hyperalgesia in the hind paw within just seven days. The differing rate of onset of hyperalgesia in these two models may be caused by the difference in the toxicity of the two drugs, or the different anatomical sites and testing methods used.

Besides altering pain sensitivity in otherwise-healthy rats (Joseph et al., 2004) and

in HIV-positive patients (Dalakas, 2001; Moyle & Sadler, 1998; Simpson & Tagliati, 1995), stavudine also may decrease the overall wellbeing of HIV-positive patients by causing other side-effects, including gastrointestinal disturbances, pancreatitis and hepatitis (Montessori et al., 2004). The effects of stavudine administration on the general condition of otherwise-healthy rats have not been investigated extensively. Therefore, I wanted to examine whether long-term daily stavudine administration affects the overall condition of the rats, and, particularly, produces deficits resulting from neural malfunction. Consequently I investigated the effect of daily stavudine administration on body mass, food intake and voluntary wheel running activity. Although stavudine administration resulted in mechanical hyperalgesia in rats within three weeks, I found that prolonged oral administration of stavudine had no adverse effects on the overall condition of the rats. Voluntary wheel running activity, appetite and growth did not differ between rats receiving stavudine and placebo. These results indicate that any adverse effects that stavudine administration may have on the general health of the rats were mild and transient, and did not affect growth and food intake over six weeks. Because stavudine administration also did not affect voluntary running activity it appears that the drug does not cause spontaneous pain in otherwise-healthy rats, or that spontaneous pain induced by stavudine administration is insufficient to affect the rats' ability or desire to run. Stavudine also did not cause other neurological deficits which would affect running activity.

The observation that stavudine administration caused hyperalgesia without affecting

the general condition of the rats is in agreement with the findings of Joseph et al. (2004), who showed that, although a single injection of stavudine induced mechanical hypersensitivity in rats, stavudine did not alter the physical appearance or open field behaviour of the rats. Warner and colleagues (1995) also found that rabbits administered stavudine orally once daily for 24 weeks did not exhibit signs of neurological damage, as assessed by the rabbits' hindleg movements. I have shown now, in a more extensive study of the effects of stavudine on the general welfare of rats, that stavudine-induced hyperalgesia is not associated with changes in motor function and voluntary activity, or with alterations in the rats' food intake and growth.

After establishing a rat model of stavudine-induced hyperalgesia, I wanted to investigate possible mechanisms of the hyperalgesia caused by oral stavudine administration in this model. While several studies have shown that NRTI-induced damage to peripheral nerve fibres may contribute to the heightened sensitivity to pain observed in animals administered NRTIs (Anderson et al., 1992; Bhangoo et al., 2007; Feldman et al., 1992; Patterson et al., 2000; Schmued et al., 1996; Wallace et al., 2007b), the role of the central nervous system, and the spinal cord in particular, in the development of NRTI-mediated pain hypersensitivity is unclear. Rat models of other peripheral neuropathic pain, including spared nerve injury, chronic constriction injury and spinal nerve ligation, are associated with neuronal death in the dorsal horn (Scholz et al., 2005). Neural damage initiates macrophage activation and is accompanied by an increase in central pro-inflammatory cytokines

and chemokines (Keswani et al., 2002; McArthur et al., 2005; Pardo et al., 2001), which may contribute to neuropathic pain (Cherry et al., 2003). Wallace et al. (2007b) recently showed that repeated systemic injection of zalcitabine to rats resulted in a modest increase in microglial and astrocyte activity in the dorsal horn, and a limited effect on dorsal root ganglion phenotype. The role of spinal neuronal damage in NRTI-induced neuropathy remains unexplored. Consequently, as stavudine is known to cause nerve damage *in vitro* (Bodner et al., 2004; Cui et al., 1997; Keswani et al., 2004), in my second study I examined whether oral administration of stavudine induces apoptosis or necrosis of spinal neurones in rats. I found that hyperalgesia that develops with chronic daily oral administration of stavudine to rats is not accompanied by spinal neuronal apoptosis or necrosis.

In addition to neuronal death in the dorsal horn (Scholz et al., 2005), other types of neuropathic pain are associated with an increase in pro-inflammatory cytokine concentrations in the spinal cord (DeLeo et al., 1996; DeLeo et al., 1997; Murphy et al., 1995; Ohtori et al., 2004; Wieseler-Frank et al., 2005). Cytokines also are thought to play a role in NRTI-induced pain (Pardo et al., 2001) and chemokines have been implicated in the progression of zalcitabine-induced pain hypersensitivity (Bhangoo et al., 2007; Wallace et al., 2007b). Thus I investigated whether daily oral administration of stavudine causes spinal release of the pro-inflammatory cytokine IL-6, which is increased in the spinal cord in other rat models of neuropathic pain (DeLeo et al., 1996; Murphy et al., 1995), and the chemokine CINC-1, which is involved in the development of other types of pain (Loram et al., 2007a; Loram et

al., 2007b). While few studies have examined concentrations of both IL-6 and CINC-1 in the spinal cord following central or peripheral nerve injury, I have found that stavudine administration resulted in an increase in the concentration of CINC-1 in the spinal cord, as assessed after six weeks of daily administration, up to three weeks after the rats developed hyperalgesia, while IL-6 concentration was unchanged throughout the six weeks of stavudine administration. These results are unique for any rat model of hyperalgesia and I am the first to show that the chemokine CINC-1 may play a role in the maintenance of stavudine-induced mechanical hyperalgesia, confirming the importance of chemokines in NRTI-induced pain hypersensitivity in rats (Bhangoo et al., 2007; Wallace et al., 2007b). The pro-inflammatory cytokine IL-6 however is unlikely to be involved in the development or the maintenance of mechanical hyperalgesia caused by prolonged stavudine administration, although this cytokine may have been elevated in the spinal cord before the onset of the hyperalgesia.

Besides causing pain hypersensitivity in otherwise-healthy rats (Joseph et al., 2004; Weber et al. 2007) and peripheral neuropathy and pain in HIV-positive patients (Dalakas, 2001; Moyle & Sadler, 1998; Simpson & Tagliati, 1995), stavudine administration, in HIV-positive patients, also is associated with other adverse events, such as lipodystrophy, the fat redistribution characterised by peripheral fat loss and central fat accumulation (Lechelt et al., 2007), and lactic acidosis caused by mitochondrial dysfunction (Montessori et al., 2004). In HIV-positive patients these side effects frequently are associated with increased plasma pro-inflammatory

cytokine concentration, decreased plasma adiponectin concentration (Jones et al., 2005; Lindegaard et al., 2004) and increased plasma lactate levels (Brew et al., 2003; Geddes et al., 2006; Haugaard et al., 2005) respectively.

Although plasma adiponectin (Maisonneuve et al., 2004) and lactate (Igoudjil et al., 2007; Lewis et al., 2005; Note et al., 2003) levels previously have been shown to remain unchanged in mice administered oral stavudine once daily, in two of these studies stavudine only was administered for two weeks (Igoudjil et al., 2007; Note et al., 2003), which may have been too brief a time for significant adverse events of stavudine administration to occur, falling one week short of the time taken for hyperalgesia to develop with oral stavudine administration to rats in my study. Studies examining the effects of stavudine administration on plasma adiponectin and lactate concentrations in mice also did not include data on changes in nociception caused by stavudine. Therefore it is unknown whether stavudine indeed causes pain hypersensitivity in mice and, as plasma cytokine, adiponectin and lactate levels have not been measured in rats administered oral stavudine, whether any changes in the concentrations of these variables correspond to the heightened sensitivity to pain induced by stavudine in rats. Thus, in my third study, to investigate whether a systemic inflammatory response or metabolic dysregulation is responsible for the hyperalgesia induced by stavudine in rats, I determined whether plasma adiponectin, lactate, CINC-1 and IL-6 concentrations were altered in rats administered daily stavudine. I have found that daily oral administration of 50 $mg.kg^{-1}$ stavudine to rats for three or six weeks did not alter plasma concentrations

of adiponectin, lactate, CINC-1 and IL-6. These findings show that, just as stavudine did not affect growth, appetite and voluntary running activity in rats, stavudine also had limited adverse effects on mitochondrial bioenergetics and fat distribution, at least as reflected in plasma adiponectin concentration, and that circulating levels of lactate, adiponectin, and the pro-inflammatory cytokines CINC-1 and IL-6 are unlikely to be involved in the development or maintenance of stavudine-induced hyperalgesia.

Although I successfully developed a rat model of stavudine-induced hyperalgesia, I was unable to determine the underlying mechanisms of this hyperalgesia. Stavudine administration did not result in spinal neuronal apoptosis or necrosis, did not cause alterations in spinal secretion of the pro-inflammatory cytokine IL-6, and did not induce peripheral release of adiponectin, lactate, IL-6 or CINC-1. The chemokine CINC-1 only was elevated in the spinal cord following six weeks of daily oral stavudine administration, which indicates that spinal CINC-1 may be involved in the maintenance of stavudine-induced hyperalgesia, but does not appear to play a role in the development of this hyperalgesia.

It is possible that the hyperalgesia induced by stavudine is the result of peripheral nerve damage caused by the drug. Stavudine administration results in the loss of peripheral neurones in HIV-positive patients (Cherry et al., 2003; Pardo et al., 2001; Polydefkis et al., 2002), and repeated systemic injections of zalcitabine to rats resulted in decreased intra-epidermal nerve fibre density (Wallace et al., 2007b).

NRTI administration to animals also is associated with changes in peripheral neurone morphology, such as myelin splitting and demyelination, and changes in nerve conduction velocity (Anderson et al., 1992; Bhangoo et al., 2007; Feldman et al., 1992; Patterson et al., 2000; Schmued et al., 1996). Similarly, in my studies, stavudine administration to rats may have caused damage to peripheral neurones, which may result in the release of excitatory amino acids, prostaglandins and nitric oxide from activated glial cells (Wieseler-Frank et al., 2005) and cause up- or down-regulation of genes responsible for the excitability of surrounding neurones (Woolf, 2004), inducing the heightened sensitivity to nociceptive input I observed (Wieseler-Frank et al., 2005; Woolf, 2004) following stavudine administration to rats.

The fact that I found no effect of stavudine administration on voluntary wheel running activity, food intake and growth, no effect on spinal neuronal viability, no effect on peripheral release of adiponectin, lactate, and cytokines and only a limited effect of stavudine administration on the production of spinal pro-inflammatory cytokines indicates that stavudine administration to rats is not associated with the numerous side-effects common to HIV-positive patients on stavudine-containing therapy. It is thought that HIV-infection may be necessary for toxic neuropathy to develop (Keswani et al., 2002; Wallace et al., 2007b). The results of my PhD indicate that HIV-infection also may be necessary for other side-effects of stavudine therapy, such as lipodystrophy and lactic acidosis, to occur. The underlying HIV infection and accompanying immune suppression may enhance the side effects of

NRTI therapy, such that these adverse effects are more pronounced in HIV-positive patients taking stavudine-containing therapy than in rats administered stavudine in the absence of HIV infection. Furthermore, the adverse effects of stavudine treatment may be enhanced by other antiretroviral drugs, particularly other NRTIs, which are prescribed as part of HAART to HIV-positive patients. As all antiretroviral drugs are associated with side effects, the combination of such agents is likely to cause a greater degree of toxicity in HIV-positive patients than the administration of only stavudine to otherwise-healthy rats.

As stavudine administration to HIV-positive patients is associated with numerous, often severe, adverse events, which may result in anxiety and depression and decrease quality of life (Larue et al., 1997; Newshan et al., 2002; Ownby & Dune, 2007), stavudine, and other NRTIs, are assumed to be intrinsically toxic. In this thesis I have shown that attaching intrinsic neurotoxicity to NRTIs may have been without foundation, as the neurotoxicity of these drugs in HIV-positive patients may result largely from the HIV/treatment interaction. Indeed Monif et al. (2007) found that a low dose (10 mg) of stavudine administered to healthy volunteers for eight days had no adverse effects on the general wellbeing of the volunteers. Although the dose of stavudine taken by the healthy volunteers was lower than that normally administered to HIV-positive patients and the duration of stavudine exposure was very brief, the results of this study indicate that stavudine may not be as toxic as previously thought and that an underlying HIV infection may be necessary for the numerous, severe side-effects of stavudine therapy to occur. This

observation may explain the difficulty of developing robust animal models of NRTI-induced neuropathy and neuropathic pain without concurrent HIV-infection.

Wallace and colleagues (2007b) recently showed that, while zalcitabine administration to rats had only a limited effect on dorsal root ganglion phenotype and glial cell activity in the spinal cord, these effects were exacerbated significantly by concurrent gp120 administration. To clarify the intrinsic toxicity of NRTIs, it is necessary to further examine the effects of NRTI administration in otherwise-healthy animals. It also is important to compare the effects of different NRTIs and to evaluate the effects of NRTI combinations in otherwise-healthy animals, which may be achieved using the model I have described here. However, to more accurately assess the adverse effects of antiretroviral drugs in HIV-positive patients, it is more appropriate to examine the mechanisms of NRTI-induced toxic neuropathy and neuropathic pain in animals concomitantly administered gp120, to mimic HIV infection.

CHAPTER SIX

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