

**THE ROLE OF THE IMMUNE SYSTEM IN
NEVIRAPINE INDUCED HEPATOTOXICITY IN A
RAT MODEL**

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ABSTRACT

Nevirapine (NVP) is an antiretroviral agent used for the prophylaxis and treatment of HIV/AIDS. Unfortunately its use is associated with severe hypersensitivity reactions and hepatotoxicity, of which the mechanism remains unclear. It was postulated to be immune mediated, as shown by recent reports that several drugs have been associated with the induction of toxicity by immune activation. Therefore, the role of the immune system in nevirapine induced hepatotoxicity was investigated here.

A high performance liquid chromatography (HPLC) assay for the determination of nevirapine in a small plasma volume was developed. Sample preparation involved protein precipitation with perchloric acid, followed by solid phase extraction on C₁₈ cartridges. The mobile phase was tetraethylammoniumphosphate (TEAP) buffer and acetonitrile (60:40, v/v) and was run over a Luna C₁₈ (4.60 x 150 mm) 5 μ analytical column at 1 ml/min. The eluent was detected by UV at 210 nm. Nevirapine and chlorzoxazone (internal standard) eluted at 2.6 and 5.2 minutes, respectively. The average 5 day calibration curve (0 – 10 μ g/ml) was linear with a regression equation of $y = 0.012x + 0.051$, and the correlation coefficient (r^2) was 0.9985. The method was successfully used to measure nevirapine concentrations in rat plasma.

The role of the immune system in nevirapine induced hepatotoxicity was investigated using an SD rat model. Rats were orally administered with nevirapine (200 mg/kg) after sub-clinical immune stimulation with a bacterial lipopolysaccharide (LPS; 2.9×10^6 E.U./kg), intraperitoneally. Blood was analysed for ALT, IFN- γ , IL-2, TNF- α , full blood count and nevirapine concentrations. A piece of liver was sent for histopathology. The corresponding controls received saline instead of nevirapine or LPS. Blood samples were taken at 6 and 24 hours after single dose administration of S+NVP, LPS+S and

LPS+NVP (acute phase), and 24 hours after single dose administration of LPS or saline to animals receiving nevirapine daily for 7, 14 and 21 days (chronic phase).

Nevirapine caused hepatotoxicity up to 7 days and progressively increased IL-2, IFN- γ and TNF- α levels, as well as the lymphocyte count over the 21 days. Nevirapine induced hepatotoxicity was characterised by apoptosis and degeneration changes, while for LPS it was cell swelling, leukostasis and portal inflammation. Co-administration of LPS+NVP attenuated nevirapine induced hepatotoxicity, exhibited lower IL-2 and IFN- γ levels, with increased neutrophil and lymphocyte count, and nevirapine concentrations.

In conclusion, nevirapine stimulated the immune system, leading to hepatotoxicity that was prevented by co-administration with LPS, and this implies that manipulation of the immune system may help to prevent nevirapine induced hepatotoxicity.

DECLARATION OF INDEPENDENT WORK

I, Zanelle Bekker, hereby declare that the dissertation hereby submitted by me for the M.Med.Sc degree in Pharmacology at the University of the Free State is my own independent work and has not previously been submitted by me at another university or faculty for admission to a degree or diploma or any other qualification. I furthermore cede copyright of the dissertation to the University of the Free State.

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SUPERVISOR'S DECLARATION

I, Professor A. Walubo, the supervisor of the dissertation entitled: The role of the immune system in nevirapine induced hepatotoxicity in a rat model, hereby certify that the work in this project was done by Zanelle Bekker at the Department of Pharmacology, University of the Free State.

I hereby approve submission of this dissertation and also affirm that this has not been submitted previously to this or any other institution for admission to a degree or any other qualification.

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ABBREVIATIONS

| | |
|---------------|--|
| AIDS | acquired immune deficiency syndrome |
| ALT | alanine aminotransferase |
| ALP | alkaline phosphatase |
| APC | antigen presenting cell |
| ARV | antiretroviral |
| AST | aspartate aminotransferase |
| B cell | B lymphocyte |
| Cal | calibration |
| CD | cluster of differentiation |
| CMI | cell mediated immunity |
| CNS | central nervous system |
| CV | coefficient of variation |
| CYP450 | cytochrome P450 |
| CYP2B6 | cytochrome P450 enzyme 2B6 |
| CYP3A4 | cytochrome P450 enzyme 3A4 |
| CZN | chlorzoxazone |
| DNA | deoxyribonucleic acid |
| EDTA | ethylamine-diamine-tetraacetic acid |
| ELISA | enzyme-linked immunosorbent assay |
| EU | endotoxin units |
| FDC | follicular dendritic cell |
| GM-CSF | granulocyte macrophage colony stimulating factor |
| HBV | hepatitis B virus |
| HCV | hepatitis C virus |
| HIV | human immunodeficiency virus |
| HPLC | high performance liquid chromatography |
| IFN | interferon |
| IgE | immunoglobulin E |

| | |
|-----------------|---|
| IL | interleukin |
| IL-2R | interleukin-2 receptor |
| IS | internal standard |
| LC-MS-MS | liquid chromatography tandem mass spectrometry |
| LPS | lipopolysaccharide |
| MCH | mean corpuscular haemoglobin |
| MCHC | mean corpuscular haemoglobin concentration |
| MCV | mean corpuscular volume |
| MHC | major histocompatibility complex |
| NAPQI | N-acetyl-benzoquinoneimine |
| NK cell | natural killer cell |
| NKT cell | natural killer T cell |
| NNRTI | non-nucleoside reverse transcriptase inhibitor |
| NO | nitric oxide |
| NRTI | nucleoside and nucleotide reverse transcriptase inhibitor |
| NVP | nevirapine |
| PI | protease inhibitor |
| PKR | protein kinase R |
| RNA | ribonucleic acid |
| Rx | treatment |
| S | saline |
| SD | Sprague-Dawley |
| SD | standard of deviation |
| T cell | T lymphocyte |
| Tc cell | cytotoxic T cell |
| TCR | T cell receptor |
| TEAH | tetraethylammoniumhydroxide |
| TEAP | tetraethylammoniumphosphate |
| Th cell | helper T cell |
| TLC | thin layer chromatography |

| | |
|------------|------------------------|
| TLR | toll like receptor |
| TNF | tumour necrosis factor |
| UV | ultraviolet |

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GENERAL INTRODUCTION OF NEVIRAPINE INDUCED HEPATOTOXICITY

The human immunodeficiency virus and/or the acquired immune deficiency syndrome (HIV/AIDS) have become a leading cause of death in all age groups. It attacks the immune system and causes an initial overstimulation, and eventually a depletion of the immune function. Due to reduced immune function the body becomes susceptible to many opportunistic infections, leading to the development of AIDS. The development of drugs for prophylaxis and treatment of HIV has been researched extensively over the past few years, but as yet there is no successful cure for the pandemic. Currently there are five classes of antiretroviral (ARV) drugs, each classified according to their mechanism of action. They are: nucleoside and nucleotide reverse transcriptase inhibitors (NRTIs), non-nucleoside reverse transcriptase inhibitors (NNRTIs), protease inhibitors (PIs) and fusion inhibitors.

Nevirapine is a NNRTI used for the prophylaxis and treatment of HIV/AIDS. Unfortunately its use is associated with severe hypersensitivity reactions such as skin rash and hepatotoxicity, hampering its use in patients who need the therapy, particularly for prophylaxis (Johnson *et al.*, 2002).

Hepatotoxicity occurs within the first six weeks of nevirapine treatment in HIV/AIDS patients with a CD4 count greater than 250 cells/mm³ (female) or 400 cells/mm³ (male) (Boehringer Ingelheim Pharmaceuticals, 2007). Whereas the mechanism of nevirapine induced hepatotoxicity remains unknown, it was postulated to be immune mediated (Dieterich *et al.*, 2004; Stern *et al.*, 2003). Such an association has already been proven in animal models for nevirapine induced skin reactions (Popovic *et al.*, 2006; Shenton *et al.*, 2003). Likewise, several drugs have shown to induce hepatotoxicity by activation of the immune system, namely diclofenac (Deng *et al.*, 2006), paracetamol (Jaeschke, 2005; Liu and Kaplowitz, 2006 and 2007), ranitidine

(Luyendyk *et al.*, 2003) and trovafloxacin (Shaw *et al.*, 2007). Already, the search is on for chemokine inhibitors as antidotes for paracetamol induced hepatotoxicity (Gardner *et al.*, 2003).

The activation of the immune system has also been demonstrated in the pathogenesis of some diseases, such as viral hepatitis due to hepatitis B virus (HBV) and hepatitis C virus (HCV) infection. Here, an activated cell mediated immune response was incriminated for the liver damage (Holt and Ju, 2006; Priimägi *et al.*, 2005). This was evidenced by a rise in type 1 (T_h1) pro-inflammatory cytokines, *i.e.*, interleukin-2 (IL-2), interferon-gamma (IFN- γ) and tumour necrosis factor-alpha (TNF- α), and type 2 (T_h2) anti-inflammatory cytokines, *i.e.*, interleukin-4 (IL-4), interleukin-6 (IL-6) and interleukin-10 (IL-10; Kulmatycki and Jamali, 2005). In fact, HBV and HCV are proven risk factors for nevirapine induced hepatotoxicity (Patel *et al.*, 2004; Martinez *et al.*, 2001).

This implies that increased stimulation of the cell mediated immune response in HIV/AIDS patients may predispose patients to nevirapine induced hepatotoxicity. However, the fact that it takes some weeks to develop liver injury means that nevirapine itself plays a role in the initiation of the lesion. Recently, it was reported that nevirapine induced hepatotoxicity was associated with enzyme induction for CYP3A and CYP2B6, but the two enzymes were not involved in the process (Walubo *et al.*, 2006). It was then proposed that a factor other than CYP450 enzymes is involved. Here, it was envisaged that nevirapine activates the cell mediated immune response, which leads to liver injury that is then propagated by the drug itself or the immune system. As such, a study on the role of the immune system in nevirapine induced hepatotoxicity was undertaken with the hope that it will shed light on the mechanism and possible modes of therapy for nevirapine toxicity.

LITERATURE REVIEW

PART I: AN OVERVIEW OF NEVIRAPINE INDUCED HEPATOTOXICITY

2.1 Pharmacology of nevirapine

Nevirapine (VIRAMUNE®) is a potent non-nucleoside reverse transcriptase inhibitor (NNRTI) used for treatment of HIV-1, where it is used in combination with other anti-retroviral agents (Cheeseman *et al.*, 1993). It is also used as monotherapy for the prevention of mother to child HIV-1 transmission (Mirochnick *et al.*, 2000). Nevirapine is a benzodiazepine derivative (Figure 2.1), a member of the dipyridodiazepinone class of compounds with a molecular weight of 266.3 g/mol (Mirochnick *et al.*, 2000). It is a weak base with a pKa of 2.8 and is highly lipophilic (Cheeseman *et al.*, 1993). The drug is an off-white powder that is currently available as a 200 mg tablet as well as a 10 mg/ml oral suspension.

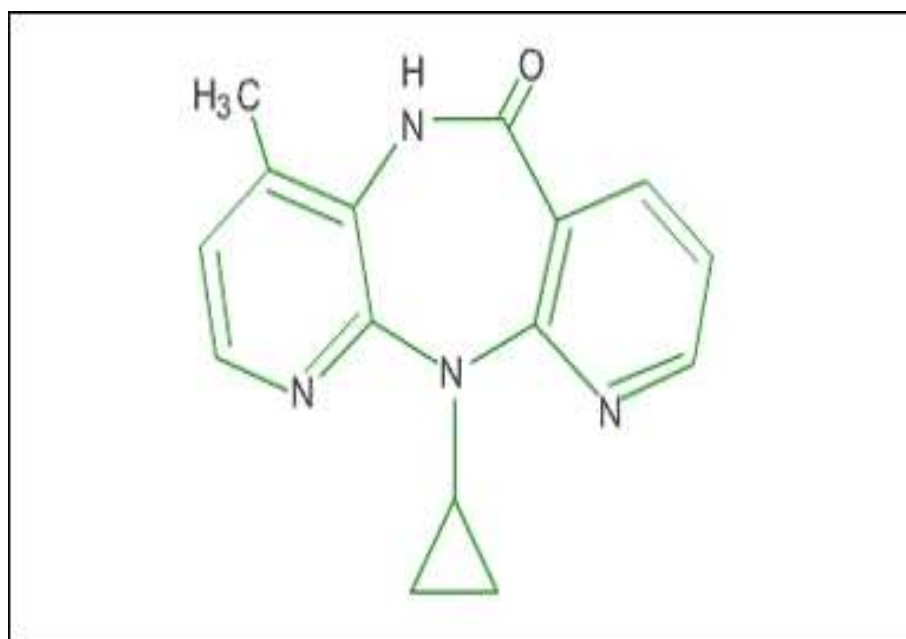


Figure 2.1: Chemical structure of nevirapine (Available from: <http://www.matrixlabsindia.com/inside/images/products/Nevirapine.gif>)

Nevirapine interferes with the binding potential of reverse transcriptase, an enzyme that transcribes HIV/viral RNA to DNA. It is a highly selective, non-competitive inhibitor of the HIV reverse transcriptase enzyme. It binds to a site adjacent to the active site of reverse transcriptase leading to a conformational change of the enzyme and consequent failure in the synthesis of complimentary viral DNA from viral RNA (Howland and Mycek, 2006).

Nevirapine is well absorbed after oral administration and absorption is not affected by food or antacids. The drug is highly lipophilic and therefore enters the fetus and mother's breast milk with ease. It is also widely distributed in the tissues, including the CNS. Nevirapine is metabolised by CYP3A4 and CYP2B6 in the liver (Howland and Mycek, 2006). The CYP3A4 and CYP2B6 enzymes are also induced by nevirapine, thereby leading to autoinduction of the drug. This autoinduction results in a decrease in the half-life of nevirapine from 45 hours, after a single dose, to 25 – 30 hours, after repeated dosing (Boehringer Ingelheim Pharmaceuticals, 2007).

2.2 Toxicity of nevirapine

The most common adverse reactions associated with nevirapine are hepatitis, Stevens-Johnson syndrome, toxic epidermal necrolysis and other skin reactions (Boehringer Ingelheim Pharmaceuticals, 2007). Other concerns include fat redistribution and the immune reconstitution syndrome.

2.2.1 Skin reactions

Nevirapine is commonly associated with a mild to moderate rash in 13% of patients. The more severe and life threatening skin reactions such as Stevens-Johnson syndrome, toxic epidermal necrolysis and hypersensitivity are associated with symptoms such as rash (grade 3 and 4), constitutional findings, organ dysfunction and rhabdomyolysis. These adverse events occur within the first six weeks of treatment and can be fatal (Boehringer Ingelheim Pharmaceuticals, 2007).

2.2.2 Hepatotoxicity

This adverse event usually emerges within the first six weeks of treatment and can lead to severe liver damage and/or liver failure (Haehl, 2000). Other hepatic events include fulminant and chronic hepatitis, and hepatic necrosis. Patients experience symptoms such as fatigue, malaise, anorexia, nausea, jaundice, liver tenderness/hepatomegaly and abnormal serum transaminase levels (Boehringer Ingelheim Pharmaceuticals, 2007).

2.2.3 Postulations concerning the mechanism of nevirapine toxicity

In general the above-mentioned adverse events have hampered the use of nevirapine in HIV patients. Therefore, nevirapine use is restricted unless the benefit of the drug outweighs the risk. The exact mechanism of nevirapine induced hepatotoxicity is currently unknown, although many postulations have been made. The toxicity usually occurs within the first six weeks of treatment in female patients with a CD4 count greater than 250 cells/mm³ and in male patients with a CD4 count greater than 400 cells/mm³ (Boehringer Ingelheim Pharmaceuticals, 2007). Nevirapine toxicity is characterised as an idiosyncratic drug reaction, *i.e.*, it is not dose related, nor does it occur to the same extent in every patient (Shenton *et al.*, 2003).

It was postulated that enzyme induction might play a role in nevirapine induced toxicity. As mentioned earlier (Section 2.1), nevirapine is metabolised by the cytochrome P450 enzymes 3A4 and 2B6, and at the same time, it induces both of these enzymes. Induction occurs from week 2 – 4 of treatment, as does toxicity. Therefore it was thought that enzyme induction contributed to the hepatotoxicity. However, it was found that there was no link between the two incidents (Walubo *et al.*, 2006).

Recent studies in animals have indicated that there is a link between nevirapine toxicity and the immune system, especially regarding skin reactions (Popovic *et al.*, 2006). In one study, there was a prominent increase in total lymphocyte and macrophage cell counts in the nevirapine treated rats when compared to the control rats (Popovic *et al.*, 2006). They postulated

that macrophages take up nevirapine and its metabolites as well as the modified skin tissue proteins, and present them on the surface of their major histocompatibility complex (MHC) I molecules for T cells to recognise and trigger the immune response (Popovic *et al.*, 2006).

In another study in which nevirapine induced skin rash was researched, other indicators of the immune-mediated mechanism were observed (Shenton *et al.*, 2005). Researchers found that there was a delay between the initiation of nevirapine treatment and the onset of skin rash, the presence of perivascular mononuclear cell infiltrates in the dermis of rash patients, and a decrease in time to onset, as well as an increase in the severity of rash on nevirapine rechallenges (Shenton *et al.*, 2005).

The observations imply that the immune system contributes to nevirapine induced toxicity, but this has not been researched for hepatotoxicity.

PART II: AN OVERVIEW OF THE IMMUNE SYSTEM

2.3 The immune system

The immune system is a collection of mechanical, chemical and biological barriers which interact to produce a collection of mechanisms to protect the body against disease. It is very important for the immune system to distinguish between foreign particles and/or pathogens. As HIV, or any foreign particle for that matter, enters the body, a series of immunological responses and defences are triggered, each with increasing specificity. The immune system can be divided into two responses, namely, the innate immune response and the adaptive immune response. Both innate and adaptive immunity play a key role in the attempt to protect the body against the invading virus. Table 2.1 briefly describes the major components of both the innate and adaptive immune systems.

Table 2.1: Components of the immune system

| Innate immune response | Adaptive immune response |
|--|--|
| Response is non-specific | Pathogen and antigen specific response |
| Exposure leads to immediate maximal response | Lag time between exposure and maximal response |
| No immunological memory | Immunological memory |

(Available from: http://en.wikipedia.org/wiki/Immune_system)

2.4 Innate immunity

Innate immunity is the body's first line of defence against any foreign invader. It is a non-specific response which keeps the viral spreading under control until the more specific adaptive immune responses can provide protection (Sherwood, 2004). The response is often described as "generic" (Alberts *et al.*, 2002) as it does not confer long-lasting immunity, although it is the most dominant system of host defence in most organisms (Litman *et al.*, 2005). Here, inflammation and cells of the innate immune response will be discussed.

2.4.1 Inflammation

Inflammation is described as one of the first responses of the immune system to infection (Kawai and Akira, 2006). It is characterised by four very prominent symptoms, *i.e.*, redness (*rubor*), heat (*calor*), swelling (*tumor*) and pain (*dolor*). A fifth symptom was later added, known as dysfunction of organs or *functio laesa* (Rosenburg *et al.*, 1999). Inflammation is a complex response of the vascular tissues to harmful stimuli, therefore its goals are to remove the injurious stimulus and to initiate the healing process. There are two types of inflammation, namely acute and chronic inflammation. Table 2.2 illustrates the differences between acute and chronic inflammation.

Table 2.2: A comparison between acute and chronic inflammation

| | Acute inflammation | Chronic inflammation |
|--------------------------|--|--|
| Causative agent | Pathogens, injured tissues | Persistent, acute inflammation due to non-degradable pathogens, persistent foreign bodies/autoimmune reactions |
| Cells involved | Neutrophils | Monocytes, macrophages, lymphocytes, fibroblasts |
| Primary mediators | Vasoactive amines | Interferon- γ , growth factors, reactive oxygen species, hydrolytic enzymes |
| Onset | Immediate | Delayed |
| Duration | Days | Months/years |
| Outcomes | Healing, abscess formation, chronic inflammation | Tissue destruction, fibrosis |

(Available from: <http://en.wikipedia.org/wiki/Inflammation#Types>)

The ultimate goal of inflammation is to isolate and destroy pathogens and foreign particles and to clear the inflamed area for tissue repair to take place. In regenerative tissues, such as skin, bone and liver, healthy cells start to replicate rapidly in order to replace the lost cells. In nonregenerative tissues, or more specifically, nerves, the lost cells are replaced by scar tissue. Fibroblasts, a type of connective tissue cell, start secreting collagen and fill the space of the lost cells, resulting in scar tissue formation (Sherwood, 2004).

Here it is concluded that inflammation is the first response to invasion by pathogens and cell injury. It is essential in wound healing and tissue repair and it is a system which has to be regulated within tight borders.

2.4.2 Cells of the innate immune response

2.4.2 a) Phagocytes

Phagocytes are cells which ingest and destroy micro-organisms and debris by phagocytosis, e.g. macrophages, dendritic cells and neutrophils (Prescott *et al.*, 1993). Phagocytosis (Figure 2.2) is the process by which these cells engulf foreign particles by literally folding their membranes around the particle. The particle is sealed off into a vacuole known as a phagosome. Here, the phagosome is delivered to a lysosome (containing proteolytic enzymes), which then fuses with the phagosome to form a phagolysosome. Foreign particles are immediately degraded and released via exocytosis. Regarding innate immunity, phagocytosis is important in the control of inflammation.

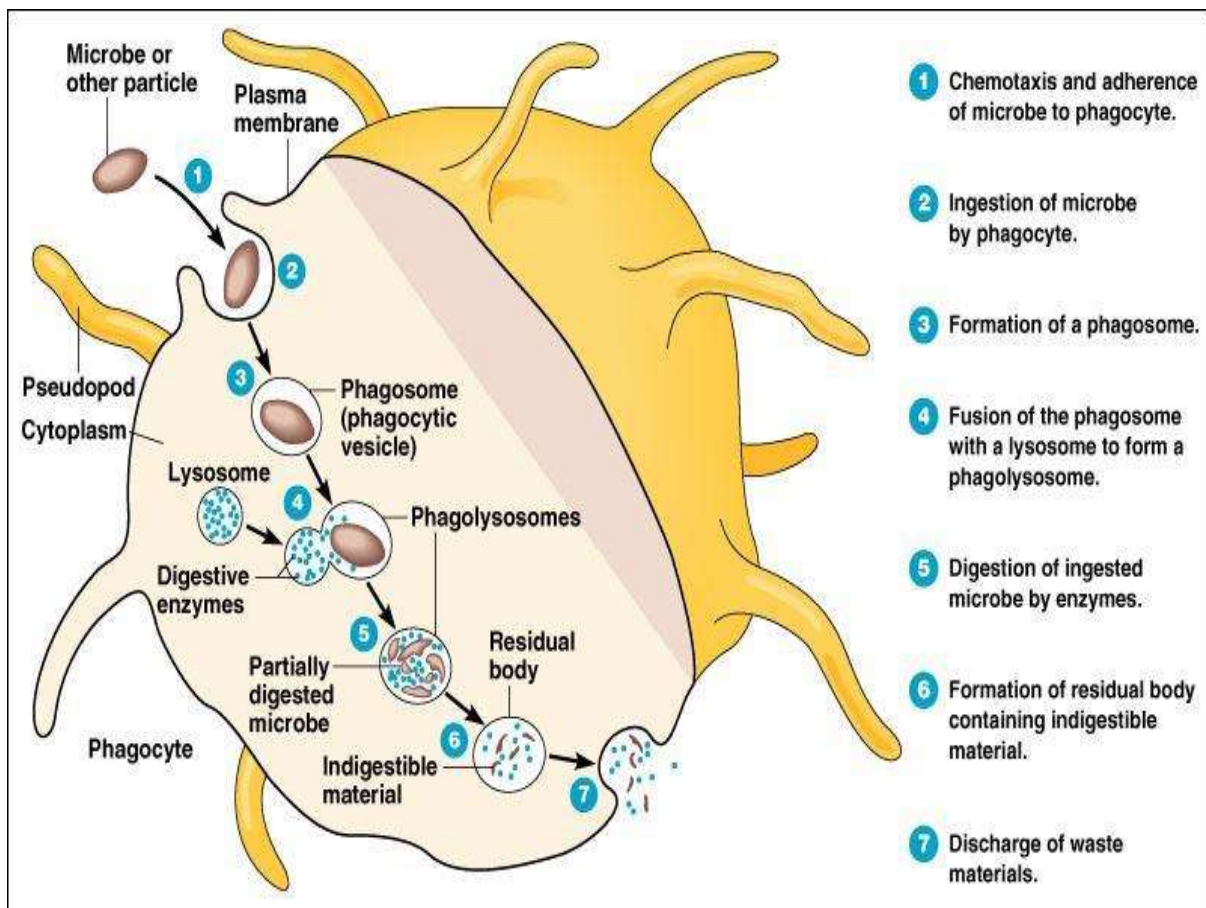


Figure 2.2: An illustration of the process of phagocytosis (Available from:

http://diverge.hunter.cuny.edu/~weigang/Images/16-08a_phagocytosis_1.jpg)

2.4.2 b) Mast cells

Mast cells are resident cells present on the skin, mucosa of the lungs and in the gastrointestinal system (Prussin and Metcalfe, 2003). They contain many granules rich in substances such as histamine and heparin, and play a major role in wound healing and pathogenic defence (Prussin and Metcalfe, 2003). Mast cells also mediate inflammation and have a fundamental role in the innate immune response. Their role in innate immunity is not well understood, but is thought to trigger the release of many cytokines and other inflammatory mediators. Mast cells contain two categories of inflammatory mediators, namely preformed mediators and newly generated mediators. Preformed mediators, such as histamine and heparin, are stored in the granules and are secreted once the mast cell is activated. In contrast, newly generated mediators are not present in the resting mast cell. They are produced during immunoglobulin E (IgE) mediated activation and are known as leukotriene C, prostaglandin D₂ and cytokines, namely tumour necrosis factor- α and interleukins-4, 5 and 6 (TNF- α , IL-4, IL-5 and IL-6; 'Stvrtinová *et al.*, 1995).

2.4.2 c) Macrophages

Macrophages are a type of phagocyte and originate from monocytes, a type of white blood cell. Monocytes are attracted to a damaged site by chemical substances through chemotaxis (Section 2.4.2 d). They enter damaged tissue through the endothelium of blood vessels and undergo a series of changes to become macrophages. Chemotaxis is triggered by stimuli such as damaged cells, pathogens, histamine and cytokines. Macrophages play a role in both innate and adaptive immunity and have a lifespan of months to years.

2.4.2 d) Neutrophils

Neutrophils are also classified as phagocytes and are the most abundant type of white blood cell in the human body. They react within an hour of tissue injury and are therefore classified as the hallmark of acute inflammation (Cohen and Burns, 2002). During acute inflammation they will, however, migrate to the inflammation site by a process called chemotaxis. Firstly,

neutrophils are slowed down in the bloodstream by selectins, an adhesion factor, causing them to marginate with the endothelium of the blood vessel. Once marginated, neutrophils adhere to the endothelium through integrins, another type of adhesion factor (Sherwood, 2004). The neutrophil leaves the blood vessel through the capillary pore in an amoebae-like fashion, in a process called diapedesis. It is then guided to the site of inflammation by chemotaxins and chemokines, such as IL-8 and IFN- α , where it survives for 1 – 2 days (Sherwood, 2004).

2.4.2 e) *Dendritic cells*

Dendritic cells are antigen presenting cells. They phagocytise pathogens and degrade their proteins, in order to express these degraded particles as antigens along with a major histocompatibility complex (MHC) so that other phagocytes still recognise them as part of the body. Once activated, dendritic cells can survive for only a couple of days. Dendritic cells are commonly known to secrete IL-12 (Reis e Sousa *et al.*, 1997), which in turn will activate the adaptive immune response.

2.4.2 f) *Basophils and Eosinophils*

Basophils are the least common white blood cells in the circulation. They contain large cytoplasmic granules and are very similar to mast cells. Once basophils are activated they degranulate to release a variety of substances such as histamine and heparin, also leukotrienes and IL-4. All these substances contribute to inflammation. Eosinophils, on the other hand, are more abundant and are also granulocyte type white blood cells. They develop and mature in bone marrow and differentiate in response to IL-3, IL-5 and granulocyte macrophage colony stimulating factor (GM-CSF; Metcalf *et al.*, 1987; Metcalf *et al.*, 1986 and Yamaguchi *et al.*, 1988). After maturation they migrate to the site of inflammation, in response to chemokines. Activated eosinophils secrete cytokines such as: IL-1, IL-2, IL-4, IL-5, IL-6, IL-8, IL-13 and TNF- α (Rothenberg and Hogan, 2006).

2.4.2 g) Natural killer cells

Natural killer (NK) cells are a form of cytotoxic cells, naturally present in the body (Sherwood, 2004). They are activated by IFN- α , β and γ , IL-2 and IL-12. In their cytoplasm small granules containing granzymes such as perforin and proteases can be found. Once released from the NK cell, perforin forms pores in the cell membrane of the target cell. Here, granzymes and other associated molecules can enter the target cell in order to induce apoptosis. Although NK cells are very effective in demolishing infected cells, they require mechanisms which enable them to distinguish between infected and uninfected cells. The exact mechanism is not known at present. In order to control their cytotoxic activity, NK cells contain “activating” and “inhibitory” receptors.

2.5 Adaptive immunity

The adaptive immune system is activated by the innate immune system and provides the ability to recognise and remember specific pathogens. It consists of highly specialised cells and processes in order to eliminate pathogenic encounters. This type of immunity is referred to as “adaptive” since the immune system is able to prepare itself for future challenges. The adaptive immune system consists of two legs, namely the cell mediated and humoral immune responses. For specific purposes of this study, only the cell mediated immune response will be discussed. The main functions of adaptive immunity include: antigen presentation, the generation of tailored responses, and the development of immunological memory.

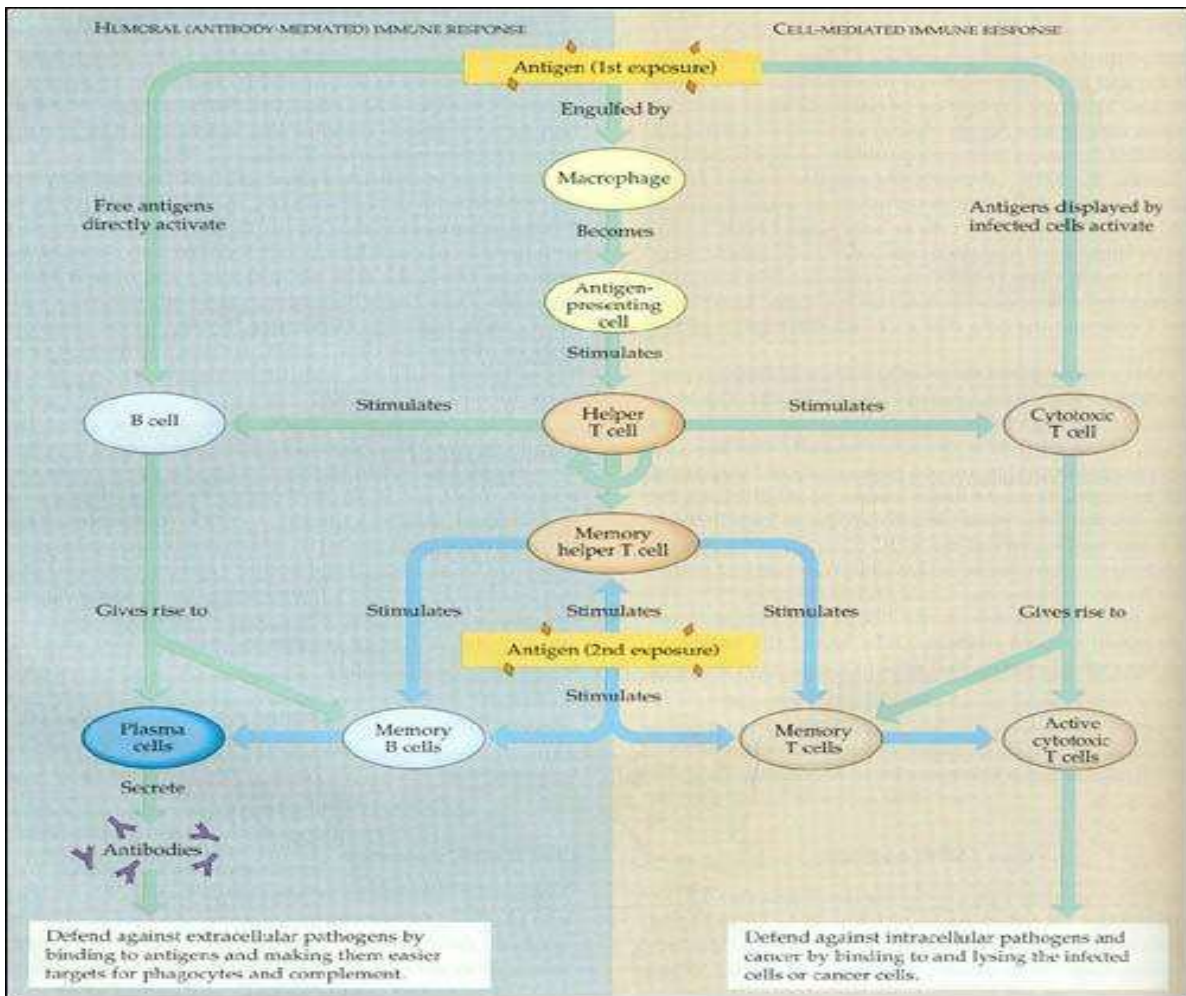


Figure 2.3: A schematic illustration of the two legs of the adaptive immune system – humoral immunity on the left and cell-mediated immunity on the right (Available from: <http://library.thinkquest.org/03oct/01254/immune.htm>)

2.5.1 Cells of the adaptive immune system

The cells of the adaptive immune response are a type of white blood cell, named lymphocytes, and are divided into two main categories: T cells and B cells. Both T and B cells are derived from pluripotential haemopoietic stem cells, they cannot be differentiated from each other until activation (Janeway *et al.*, 2001). T cells contribute to the cell mediated immune response, while the B cells are part of the humoral immune response (Figure 2.3).

2.5.1 a) T cells

T cells are derived from pluripotential cells, but migrate to the thymus where they are matured. T cells are distinguishable from other lymphocytes by the presence of T cell receptors (TCR) on their surfaces. In short, T cells are activated by two signals: firstly by the binding of the TCR and CD28 on the T cell via a signal from a MHC on an antigen presenting cell (APC, e.g. macrophage), and secondly via co-stimulation of surface receptors of the APC. There are several subsets of T cells, each with a unique function.

2.5.1 b) Helper T cells

Helper T (T_h) cells are a very unique subset of T cells. They show no cytotoxic or phagocytic activity, nor can they destroy an infected host cell or pathogens. T_h cells rather activate other immune cells, such as B cells and macrophages, in order to perform their functions. Mature T_h cells express the surface protein CD4, and are therefore also known as CD4 positive ($CD4^+$) T cells. T_h cells are activated as described above, but proliferate by secreting IL-2. They carry an IL-2 receptor to which IL-2 binds and thereby proliferates the cell. T_h cells proliferate into effector, memory, or suppressor T cells. Effector T cells further differentiate into type 1 (T_{h1}) cells, or type 2 (T_{h2}) cells (Figure 2.4).

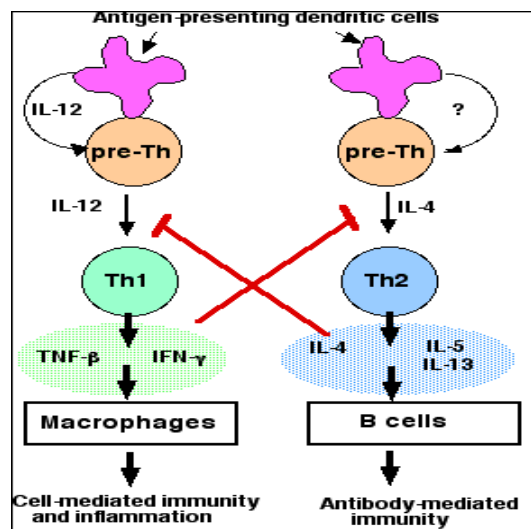


Figure 2.4: Effector T cell differentiation into T_{h1} and T_{h2} cells (Available from:

http://users.rcn.com/jkimball.ma.ultranet/BiologyPages/T/Th_Lymphokines.gif)

As illustrated in Figure 2.4, T_{h1} cells are produced from pre- T_h cells by IL-12 stimuli. T_{h1} cells then secrete pro-inflammatory cytokines TNF- β and IFN- γ in order to activate macrophages, which in turn will stimulate cell mediated immunity and inflammation. T_{h2} cells are produced in reaction to IL-4 stimuli, and secrete IL-4, IL-5 and IL-13. These anti-inflammatory cytokines stimulate B cell production, which leads to the antibody mediated immune response (Kimball, 2007).

2.5.1 c) Cytotoxic T cells

Cytotoxic T (T_c) cells are capable of inducing the death of infected or damaged cells by the release of perforin and granulysin. These cells express T cell receptors that can recognise a specific antigen peptide bound to class 1 MHC molecules and a glycoprotein called CD8. Hence, cytotoxic T cells are also referred to as CD8 positive (CD8+) T cells. Activation of CD8+ T cells occurs by the presentation of antigen peptides by MHC 1 to the T cell receptors.

2.5.1 d) Regulatory T cells

Regulatory T cells, or suppressor T cells, act to suppress activation of the immune system, and hereby homeostasis of the immune system is maintained. These cells help the immune system to differentiate between the “self” and “non-self” of the body.

2.5.1 e) Natural killer T cells

Natural killer T (NKT) cells possess properties of both T cells and NK cells (Jerud, 2006). The clinical potential of NKT cells lies in the rapid release of cytokines such as, IL-2, IFN- γ and TNF- α that promote or suppress certain immune responses.

2.6 Cytokines

Cytokines are a group of low molecular weight proteins, peptides and glycoproteins that function as signalling compounds and chemical mediators. They are released from many types of cells of the immune system in tissues undergoing defence, growth and repair (Hopkins, 2003), and play a particularly prominent role in the innate and adaptive immune responses.

It is common for a single cytokine to be produced by more than one type of cell, and also, that one cytokine may be involved in many different biological processes (Hopkins, 2003). Cytokines are redundant in the sense that several cytokines share the same activities (Hopkins, 2003).

Functionally, immunological cytokines can be divided into those that promote the proliferation and functioning of T_h1 and T_h2 cells, respectively. A T_h1 cytokine response is associated with the promotion of inflammation, while the T_h2 cytokines are responsible for clearing up inflammation (Priimägi *et al.*, 2005). Since this study is focused on how the immune system contributes to nevirapine induced hepatotoxicity, attention will be specifically paid to pro-inflammatory cytokines, IL-2, IFN- γ and TNF- α .

2.6.1 Interleukin-2



Figure 2.5: Crystalline structure of interleukin-2 (Available from:

[http://upload.wikimedia.org/wikipedia/commons/8/87/IL2_Crystal_Structure.pn](http://upload.wikimedia.org/wikipedia/commons/8/87/IL2_Crystal_Structure.png)

g)

As the first interleukin molecule to be discovered, IL-2 (Figure 2.5) has been extensively researched and its immune function established over the past few decades (Smith *et al.*, 1980). This specific cytokine is unanimously acknowledged as a “T cell growth factor” and consequently contributes immensely to homeostasis of the immune function (Gaffen and Liu, 2004). IL-2 is produced by T_h1 cells, T_c cells, some B cells and dendritic cells, and specifically targets activated T cells, B cells, NK cells and macrophages to stimulate growth and differentiation of the T cell response. It also promotes the production of TNF- α and IFN- γ by NK cells (Gaffen and Liu, 2004).

When antigens gain access to the body they are recognised as foreign by T cell receptors (TCR). As these antigens bind to the TCR, the secretion of IL-2 and expression of IL-2 receptors (IL-2R) are stimulated very rapidly. This IL-2/IL-2R interaction then stimulates the growth and differentiation of antigen specific T_c cells (Smith, 1988; Stern and Smith, 1986; Beadling *et al.*, 1993). Hereby IL-2 contributes to the development of T cell immunologic memory. IL-2 also exerts effects on cellular metabolism and glycolysis that are necessary for long-term survival of T cells (Gaffen and Liu, 2004).

IL-2 also plays an important role in the maturation of regulatory T cells (Sakaguchi, 1995; Thornton and Shevach, 1998; Thornton *et al.*, 2004), and hereby downregulates the immune response in order to prevent autoimmunity. This is considered the main nonredundant function of IL-2 (Malek, 2003). These inhibitory effects of IL-2 create a negative feedback pathway by one of two mechanisms. Firstly, in the absence of persistent antigenic stimulation IL-2 levels depletes, leading to the death of activated T cells in the cytokine deprived environment. Finally, IL-2 also initiates a proapoptotic pathway which eventually leads to programmed cell death of activated T lymphocytes (Gaffen and Liu, 2004).

2.6.2 Interferon- γ

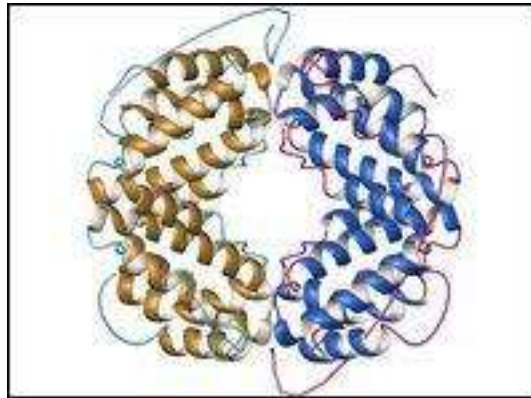


Figure 2.6: Crystalline structure of interferon- γ (Available from:

[http://www.argyllbiotechnologies.com/images/immune_overview/Human%20Interferon%20gamma%20\[200x200\].jpg](http://www.argyllbiotechnologies.com/images/immune_overview/Human%20Interferon%20gamma%20[200x200].jpg))

Interferon- γ (IFN- γ ; Figure 2.6), originally called macrophage-activating factor, is predominantly produced by NK and NKT cells as part of the innate immune response, as well as by T_h1 and T_c cells when the adaptive immune response is activated (Schoenbron and Wilson, 2007; Schroder *et al.*, 2004). During early host defence IFN- γ is produced by NK cells and APCs, while T cells become the major source in the adaptive immune response (Schroder *et al.*, 2004). Functions of IFN- γ include antiviral activity, activation of macrophages and NK cells, MHC glycoprotein enhancement, and thus foreign peptide presentation to T cells, leading to the promotion of specific cytotoxic immunity (Schroder *et al.*, 2004).

IFN- γ is important in fighting RNA virus infections, hence HIV. When HIV enters a host cell the HIV RNA is recognised as a foreign substance and therefore triggers the cell to produce IFN- γ via a toll like receptor (TLR; Sherwood, 2004). In the infected cell, TLR switches on the gene that codes for IFN- γ , which is secreted from these infected cells and binds to the plasma membranes of neighbouring healthy cells. Here IFN- γ acts as a warning signal and helps healthy cells to prepare against attack (Sherwood, 2004). The cells begin producing large amounts of protein kinase R (PKR), which start transferring phosphate groups to a translation initiation factor. This step

reduces the factor's ability to initiate translation. Viral replication is inhibited, as well as normal ribosome function within the cell.

IFN- γ has the unique ability to coordinate the transition from innate immunity to adaptive immunity. It coordinates the transition by the following mechanisms: aiding in the development of a T_h1 cell response; directly promoting B cell isotype switching; and, along with NO, regulation of local leukocyte-endothelial interactions (Schroder *et al.*, 2004). IFN- γ is indeed a remarkable cytokine responsible for many cellular programs, resulting in increased immune surveillance and immune system function.

2.6.3 Tumour necrosis factor- α

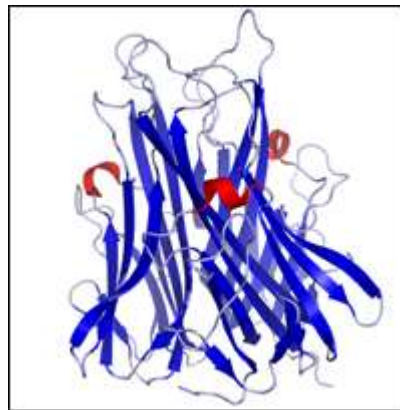


Figure 2.7: Crystalline structure of tumour necrosis factor- α (Available from: http://www.argyllbiotechnologies.com/images/immune_overview/TNFa_Crystal_Structure.rsh.png)

Tumour necrosis factor- α (TNF- α ; Figure 2.7) is a very versatile cytokine, possessing properties to stimulate growth, inhibit growth and finally to regulate itself (Murray *et al.*, 1997). It is also able to induce apoptotic cell death, inflammation and to inhibit viral replication (Locksley *et al.*, 2001). This cytokine is an acute phase protein, responsible for the initiation of a cascade of cytokines and it increases vascular permeability, thereby recruiting macrophage and neutrophils to the site of infection. Here, TNF- α is secreted by macrophages which cause blood clotting which serves to contain the infection (Janeway *et al.*, 1999). As already mentioned, TNF- α is primarily

produced by macrophages, but it is also produced by other cells such as mast cells and fibroblasts. Its release is also potently stimulated in response to lipopolysaccharide of bacterial origin (Tukov *et al.*, 2007).

TNF- α production leads to cytolysis of many tumour cell lines *in vivo*. It is also a growth factor for human fibroblasts, where it promotes the production of collagenase and prostaglandin E₂, a known inducer of fever (Ibelgauft, 2007). The proliferation of T cells is enhanced by TNF- α , and in the presence of IL-2, TNF- α stimulates the production of B cells (Ibelgauft, 2007).

Although TNF- α is required for normal immune function, over-expression of this mediator is associated with symptoms such as cachexia in tumour patients, and severe effects during Gram negative sepsis (Ibelgauft, 2007).

PART III: THE ROLE OF THE IMMUNE SYSTEM IN DRUG TOXICITY

2.7 Toxicity of the immune system

The immune system has been implicated in the pathogenesis of some diseases (Priimägi *et al.*, 2005), as well as the development of drug toxicity. Activation of the immune system has been demonstrated in viral hepatitis due to hepatitis B virus (HBV) and hepatitis C virus (HCV) infection, and both T_h1 and T_h2 cytokines were implicated (Priimägi *et al.*, 2005).

In acute viral hepatitis, a strong T_h1 response leads to the production of cytotoxic and natural killer (NK) cells, which attack and eliminate infected cells. This then leads to liver injury (Huang, *et al.*, 2006; Jacobson - Brown and Neuman, 2001).

2.8 Immune associated drug toxicity

Drug toxicity due to over-excitation of the immune system has been observed with drugs such as acetaminophen, diclofenac and penicillin. The mechanisms associated with immune stimulation range from idiosyncratic reactions to overdose and the hapten hypothesis (Holt and Ju, 2006).

During acetaminophen overdose, it was reported that the toxic metabolite, N-acetyl-p-benzoquinoneimine (NAPQI), causes the initial injury to hepatocytes (Holt and Ju, 2006). The injury triggers the immune system to activate NK cells and to produce T_h1 cytokines. Unfortunately the T_h1 cytokines cause further tissue damage by increased stimulation of the natural killer cells (Holt and Ju, 2006).

Diclofenac has been associated as an idiosyncratic drug reaction in which severe hepatotoxicity is prominent and for which the mechanism is not clear. However, recent reports have demonstrated immune activated diclofenac hepatotoxicity in an animal model (Deng *et al.*, 2006). Here, a non-toxic dose of diclofenac was administered alone, and after pre-treatment with lipopolysaccharide (LPS), an immune stimulant. Of note, the dose of LPS used was aimed at inducing non-clinical inflammation. This finding suggested that mild inflammation enhanced diclofenac hepatotoxicity, implying the involvement of the immune system (Deng *et al.*, 2006).

The hapten hypothesis suggests that reactive metabolites of drugs bind to endogenous proteins, forming immunogenic drug-protein adducts (Holt and Ju, 2006). These adducts then attract either antibodies or cytotoxic T cells. This hypothesis is applicable to drugs such as penicillin and diclofenac (Holt and Ju, 2006). However, this form of cell injury is indirect given that the body eventually dies of shock.

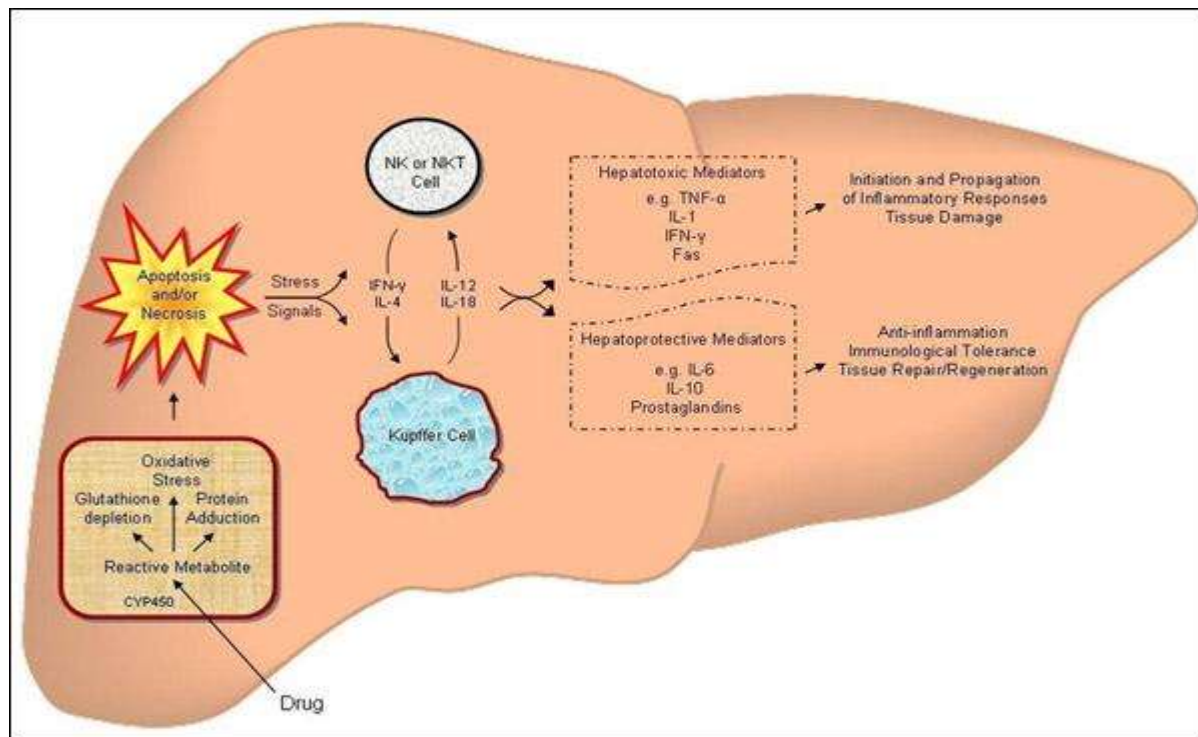


Figure 2.8: An illustration of the proposed mechanisms of drug induced liver toxicity (Available from: <http://www.aapsj.org/view.asp?art=aapsj080106>)

In general, it has been shown that in both viral infection and drug toxicity, the immune system is stimulated by direct cell injury via the T_h1 response and NK cells. Therefore it is likely that this most probably also applies to nevirapine induced hepatotoxicity.

In the same perspective, to understand the mechanism of nevirapine associated liver injury in HIV patients, one needs to understand the state of the immune system in HIV patients.

PART IV: IMMUNE RESPONSE TO HIV

2.9 The three stages of HIV infection

The clinical picture of HIV infection is categorised into three different stages, namely: primary HIV infection, the clinically asymptomatic stage, and symptomatic HIV infection. The severity of each stage is determined by the relationship between the CD4 cell count and viral load (Figure 2.9). A change in cytokine profiles during the progression of HIV infection was also reported (Klein *et al.*, 1997).

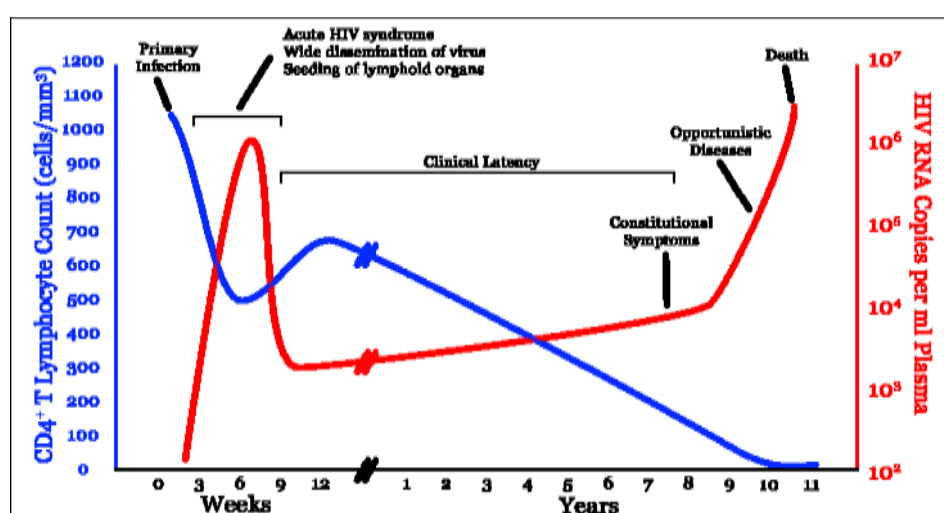


Figure 2.9: A graph of the relationship between HIV copies (viral load) and CD4 counts over the average course of untreated HIV infection (Available from: <http://en.wikipedia.org/wiki/Image:Hiv-timecourse.png>)

2.9.1 Stage I: Primary HIV infection

Stage I occurs soon after HIV transmission and may last up to 12 weeks. It corresponds to the acute clinical phase that is characterised by non-specific symptoms such as fever, rash and malaise, often leading to the misdiagnosis of HIV infection. There is rapid viral replication which reaches a peak at about 5 – 6 weeks and is associated with a rapid fall in CD4 cells which reaches trough at the same time (Figure 2.9; Denelsbeck, 2006; Piatak *et al.*, 1993). In response to the fall in CD4 cells, about 3 – 4 weeks later, the immune

system reacts by producing antibodies, CD4 cells and by activating the CD8+ cytotoxic T cells to fight the virus (Denelsbeck, 2006).

2.9.2 Stage II: Clinically asymptomatic stage

In the fourth month, the improved immune response leads to a drop in viral load, resulting in clinical latency which may last for an average of 10 years (Figure 2.9). During this stage, the immune system has reduced the viral load to a sub-clinical level. In effect there is both inflammation and viral replication. Of note, HIV is not dormant during this stage, patients just present with a low viral load (Denelsbeck, 2006). HIV continues to reproduce within lymphoid organs and a large amount of the virus becomes trapped in the follicular dendritic cells (FDC) network (Burton *et al.*, 2002).

2.9.3 Stage III: Symptomatic HIV infection

After the latency period the cell mediated immunity is lost, as indicated by a decline in CD4 numbers to an average of 50 – 100 points per year (Figure 2.9; Denelsbeck, 2006). These conditions create an optimal environment for opportunistic microbes to manifest infection and cancers to develop, especially with a CD4 count of less than 200 cells/mm³ (Denelsbeck, 2006).

2.10 Cytokines associated with the different HIV stages

The changes in the levels of CD4 cells enumerated earlier can be used to predict the status of cytokine profiles. This is because CD4 is a sign of activity of T_h1 cytokines. Therefore, stage I and II which have a high CD4 count (> 400 cells/mm³) should exhibit high T_h1 cytokines (IL-2, IFN- γ and TNF- α), while stages III and IV with a low CD4 count (< 200 cells/mm³) will exhibit low T_h1 cytokine levels, but increased T_h2 cytokines (IL-4, IL-6 and IL-10).

In fact, it was reported that there is a shift of T_h1 to T_h2 cytokine profiles during the course of HIV infection (Klein *et al.*, 1997). The loss of T cell function along with disease progression was associated with a decline in IL-2 and IFN- γ production, and significant increase in IL-4 production. Even during T_h2

cytokine production it was reported that IL-4 expression was high in stage III of HIV infection, while IL-10 expression was higher in stage IV where patients are prone to the development of AIDS (Klein *et al.*, 1997). The above-mentioned findings were also supported and verified by Clerici and Shearer (1994) and Ramalingam *et al.* (2005), while Biglino *et al.* (1996) reported low TNF- α levels during acute HIV infection.

In view of the above observations, it is hereby postulated that nevirapine induced hepatotoxicity could be an immune mediated reaction. The most plausible mechanism is an idiosyncratic reaction that is augmented by sub-clinical immune activation or improvement during HIV treatment with nevirapine.

REVIEW OF ANALYTICAL METHODS FOR DETERMINATION OF NEVIRAPINE IN PLASMA

3.0 Summary

Many analytical methods for the determination of nevirapine in plasma are described, and vary from liquid chromatography tandem mass spectrometry (LC-MS-MS) to thin layer chromatography (TLC) and high performance liquid chromatography (HPLC). All the methods reviewed for LC-MS-MS and TLC hold certain advantages over HPLC, but HPLC is still considered the “golden standard” for antiretroviral drug level measurement (Cressey *et al.*, 2007).

3.1 Liquid chromatography tandem mass spectrometry

The liquid chromatography tandem mass spectrometry (LC-MS-MS) methods reviewed were focussed on rapid and simple extraction methods of nevirapine in human plasma (Martin *et al.*, 2009 and Chi *et al.*, 2002). These LC-MS-MS assays showed to be very sensitive, accurate and precise. Also, small plasma volumes were used, which was appealing as this was one of the major objectives of our method development. Although LC-MS-MS holds many advantages and promises to quantitate nevirapine in plasma, the instrumentation used and maintenance are expensive.

3.2 Thin layer chromatography

Thin layer chromatography (TLC) is an inexpensive, simple and rapid assay, and can be used in laboratories with limited resources. The reviewed methods describe large plasma volumes (Dubuisson *et al.*, 2004), which were inappropriate for our specific goals of method development. An immunochromatographic strip test based on TLC principles was also reviewed (Cressey *et al.*, 2007). This method was unable to provide information regarding the minimum effective concentration of nevirapine (Cressey *et al.*, 2007).

3.3 High performance liquid chromatography

High performance liquid chromatography is considered the quantitative "gold standard" for antiretroviral drug level measurement (Cressey *et al.*, 2007). The use of an internal standard was rare in most of the reviewed methods. However, Ramachandran *et al.* (2006) used 3-isobutyl 1-methyl xanthine as internal standard and performed a simple liquid-liquid extraction with ethyl acetate. This method requires a sample volume of 250 μ l and detection by a diode array detector, which is not available in our setup. Van Heeswijk and co-workers (1998) also performed a liquid-liquid extraction using acetonitrile. A sample volume of 250 μ l was used and the method had a lengthy run time of 12 minutes. In addition, a reversed phase analytical column was used.

Pav *et al.* (1998) used BIRH-414 as internal standard. They reported a complex solid phase extraction method, and used a thermostated, reversed phase column for analysis. Other rapid methods included those of Hollanders *et al.* (2000) and Lopez *et al.* (2000). Both these methods have short run times and simple sample preparation by acidification of the sample with either perchloric acid (Hollanders *et al.*, 2000), or trichloroacetic acid (Lopez *et al.*, 2000). After centrifugation the supernatant was directly injected into the HPLC. Although a short run time and simple sample preparation are appealing when developing a method, especially when large numbers of samples have to be analysed, the lifetime of the analytical column is at stake.

In review of all the above-mentioned methods it was concluded that none could be adopted, owing to solvents and equipment used. However, protein precipitation by perchloric acid, as described by Hollanders and co-workers (2000), was considered as this has been successfully used in our laboratory in another experiment.

OBSERVATIONS FROM THE REVIEW

4.1 Observations from the review

In summary it was observed that:

- Nevirapine causes hepatotoxicity which hampers its use in patients who need it.
- The mechanism of nevirapine induced hepatotoxicity is unknown.
- The immune system has been implicated in the pathogenesis of viral hepatitis by HBV and HCV via cell mediated immunity.
- The immune system has been implicated in drug toxicity, also via cell mediated immunity.
- Understanding the mechanism of nevirapine induced hepatotoxicity will aid in prevention.
- Nevirapine toxicity is associated with high CD4 levels (> 400 cells/mm³), therefore there is a need to understand the role of the immune system in nevirapine induced hepatotoxicity.

4.2 Hypothesis

“The immune system has a role in nevirapine induced hepatotoxicity”

4.3 Objectives

- To develop an HPLC method for the analysis of nevirapine in plasma.
- Determining the role of the immune system during acute nevirapine induced hepatotoxicity.
- Determining the role of the immune system during chronic nevirapine induced hepatotoxicity.

DETERMINATION OF NEVIRAPINE IN A SMALL PLASMA VOLUME BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

5.0 Summary

A high performance liquid chromatography (HPLC) method for the determination of nevirapine in a small plasma volume was developed. It involved protein precipitation of 100 μ l of nevirapine spiked plasma with perchloric acid followed by centrifugation. The supernatant was purified by solid phase extraction on C₁₈ cartridges, and 40 μ l was injected into the HPLC. The sample was eluted with a mobile phase of tetraethylammoniumphosphate (TEAP) buffer: acetonitrile (60: 40, v/v) over a Luna C₁₈ (4.60 x 150 mm) 5 μ analytical column at 1 ml/min. Chlorzoxazone was used as the internal standard. Under these conditions nevirapine and chlorzoxazone eluted at retention times of 2.6 minutes and 5.2 minutes, respectively. The average calibration curve (0 – 10 μ g/ml) was linear with a regression equation of $y = 0.012x + 0.051$, and regression coefficient of $r^2 = 0.9985$. The method was used to measure nevirapine concentrations in rat plasma.

5.1 Introduction

In this chapter, a high performance liquid chromatography method for determination of nevirapine in a small plasma volume is described.

5.2 Materials and methods

5.2.1 Apparatuses

Precision and analytical balances (SPB 52 and SPB 31, Scaltec Instruments, Goettingen, Germany) were used to weigh gram and milligram amounts of reagents and drug standards, respectively. A vortex mixer (Vortex Genie 2, Scientific Industries Inc., Bohemia, NY, U.S.A) and a micro centrifuge (Minispin, Eppendorf, Hamburg, Germany) were used for mixing and quick spinning of the samples. Purification of samples was performed by solid phase extraction with C₁₈ solid phase extraction cartridges (Sep-Pak[®], Waters Corporation, Milford, MA, U.S.A) along with a solid phase extraction vacuum manifold (Visiprep-DL[™], Bellefonte, PA, U.S.A).

5.2.2 Reagents and chemicals

The analytical standard of nevirapine was obtained from Boehringer Ingelheim Pharmaceuticals, Inc. (Ridgefield, CT, U.S.A). Sodium hydroxide (NaOH), chlorzoxazone and chlorpropamide were purchased from Sigma-Aldrich Inc. (St. Louis, MO, U.S.A). The following chemicals were supplied by Merck Laboratories (Darmstadt, Germany): tetraethylammoniumhydroxide (C₈H₂₁NO), orthophosphoric acid (H₃PO₄) and perchloric acid (HClO₄). HPLC grade methanol and acetonitrile were purchased from Honeywell, Burdick and Jackson (Muskegon, MI, U.S.A). BDH Chemicals LTD. (Poole, England, U.K) supplied trichloroacetic acid (C₂HCl₃O₂). The following analytical standards were from the Department of Pharmacology Toxicology Laboratory reference substances: butriptyline, clomipramine, sulindac, dothiepin, pirtanid, sulfasomidine and sulfafurazole.

5.2.3 Preparation of mobile phase

The mobile phase was prepared from two solutions, A and B. Solution A consisted of a tetraethylammoniumphosphate (TEAP) buffer that was prepared by dissolving 2.9 g orthophosphoric acid and 15.54 g tetraethylammoniumhydroxide (TEAH) in 500 ml distilled water. Solvent B consisted of 1000 ml HPLC grade acetonitrile with 100 µl orthophosphoric acid.

The working mobile phase was prepared by mixing solvent A and B in a ratio of 60: 40 v/v. After mixing the solutions, the mobile phase was degassed with helium gas.

5.2.4 Preparation of standard solutions

First, separate standard solutions containing 1 mg/ml nevirapine and chlorzoxazone (internal standard), respectively were prepared. Both standards were further diluted to a concentration of 100 µg/ml.

Thereafter, standard plasma calibration samples were prepared by spiking 1 ml of plasma with appropriate volumes of nevirapine standard solution to obtain final concentrations of 1, 3, 5, 8 and 10 µg/ml.

5.2.5 Sample preparation

To 100 µl of the standard plasma sample 50 µl of internal standard was added, after which the sample was vortexed for 15 seconds. Then, 60 µl of 2 M (20%) perchloric acid was added to the sample in order to precipitate the proteins in solution. The sample was vortexed for 30 seconds and then centrifuged at 7026 g (13400 r.p.m) for 7 minutes. The supernatant was removed and further purified by solid phase extraction as described in the following section.

5.2.6 Column extraction

C₁₈ solid phase extraction cartridges (1 ml) were conditioned with 2 ml deionised water, followed by 2 ml HPLC grade methanol. The supernatant was placed on the column and eluted for 30 seconds. Thereafter, the column was washed with 500 µl of deionised water. The compounds were then eluted with 200 µl of an acetonitrile: water (80: 20, v/v) solution. The eluent (40 µl) was injected into the HPLC for analysis.

5.2.7 Chromatographic system

The HPLC system consisted of a Hewlett Packard model 1100, equipped with an autosampler (Waldbronn, Germany), isocratic pump (Waldbronn, Germany) and UV detector (Tokyo, Japan). Data were collected using Chem Stations software.

5.2.8 Chromatographic conditions

Chromatographic separation of nevirapine and chlorzoxazone was achieved by running the mobile phase at a flow rate of 1 ml/min. over a Phenomenex® Luna C₁₈ (4.60 x 150 mm) 5 μ analytical column, coupled to a Phenomenex® SecurityGuard™ C₁₈ (4 x 3 mm) guard column (Torrance, CA, U.S.A). Compounds were detected by UV at a wavelength of 210 nm.

5.3 Preliminary experiments

5.3.1 Preparation of standard solution of nevirapine

Nevirapine is a weak base that is highly lipophilic. Firstly, 1 mg/ml nevirapine was prepared in the mobile phase, but did not dissolve. Sodium hydroxide was added in order to dissolve the nevirapine, but this only broke down the compound. Since the drug is highly lipophilic, it was decided to use methanol to prepare a 1 mg/ml nevirapine solution.

5.3.2 Selection of an internal standard

The selection of a suitable internal standard for this method was a tedious process, since the drug had to be stable, and at the same time not be regularly prescribed. Since polypharmacy is common in HIV patients, a drug had to be selected which would not interfere with nevirapine. Therefore it was decided to choose one from a list of drugs which are rarely used.

The following drugs were tested, but with no success: butriptyline, clomipramine, sulindac, dothiepin, piretanid, chlorpropamide, sulfasomidine and sulfafurazole. Finally chlorzoxazone was tried. It dissolved well in methanol, and showed no interference with the nevirapine peak (Figure 5.1 b). Therefore, it was the preferred internal standard.

5.3.3 Sample preparation and extraction

5.3.3 a) *Liquid-liquid extraction*

As mentioned earlier, nevirapine is a basic drug. Therefore a liquid-liquid extraction using sodium hydroxide and ether was tried. The chromatogram showed interfering peaks, and the method was discarded.

5.3.3 b) *Extraction by centrifugation with trichloroacetic acid*

To nevirapine spiked plasma, 20 μ l of 50% trichloroacetic acid (Lopez *et al.*, 2000) was added in order to precipitate the plasma proteins. The sample was vortexed for 30 seconds and centrifuged for 5 minutes at 1251 *g* (2500 r.p.m). Of the supernatant 40 μ l was directly injected into the HPLC, but delivered very poor results as there was no nevirapine peak detected. Once again the method was discarded.

5.3.3 c) *Extraction by centrifugation with perchloric acid*

Based on the method of Hollanders and co-workers (2000), perchloric acid was used to precipitate proteins in solution. To plasma spiked with nevirapine, 60 μ l of 2 M (20%) perchloric acid was added, the sample was vortexed for 30 seconds and centrifuged for 5 minutes at 7026 *g* (13400 r.p.m). The supernatant was directly injected into the HPLC. Nevirapine was well extracted, but the chromatogram still showed background noise. As such, the supernatant had to be further purified. Therefore a solid phase extraction method was developed (Section 5.3.3.d).

5.3.3 d) *Solid phase extraction*

Proteins were precipitated as described in Section 5.3.3 c. C₁₈ solid phase extraction cartridges were conditioned with 2 ml deionised water and methanol, after which the supernatant was placed on the column to elute. The filtered eluent was directly injected into the HPLC, but it appeared that the compounds remained on the column as no peaks were observed.

Acetonitrile was used to elute the compounds. Nevirapine and the internal standard eluted, but the chromatogram lacked sensitivity. Thereafter a washing step was included. After conditioning of the column and filtering of the supernatant, the column was washed with 1 ml deionised water before elution. Unfortunately the results remained inconsistent.

The volume of the deionised water in the washing step was reduced. This change delivered better results, but they were still not optimal. Finally, the elution step was adjusted by eluting the compounds with acetonitrile: water (80: 20, v/v). Well eluted, sensitive peaks were observed.

The volume of the plasma sample was still debatable, as this method would be applied to measure nevirapine in rat plasma. Since rats do not have as much blood as human subjects, the sample volume was reduced even further. Here, the final conditions were set for purification of plasma samples after protein precipitation, and they proved to be reproducible.

5.4 Final conditions for sample preparation and extraction

To 100 µl of plasma spiked with nevirapine, 50 µl of internal standard was added and the sample was vortexed for 15 seconds. Thereafter the sample was acidified with 60 µl of 2 M (20%) perchloric acid in order to precipitate plasma proteins in solution. The sample was vortexed for 30 seconds, centrifuged for 5 minutes at 7026 g (13400 r.p.m) and finally purified by solid phase extraction.

A C₁₈ solid phase extraction cartridge was conditioned with 2 ml deionised water followed by 2 ml methanol. Thereafter, the sample (100 µl) was placed on the column and allowed to elute. The column was washed with 500 µl deionised water, the used test tube discarded and replaced with a fresh test tube for sample collection. Finally, the sample was eluted with 200 µl of acetonitrile: water (80: 20, v/v) and 40 µl was injected into the HPLC for analysis.

5.5 Standardization

Calibration was done by analysing plasma samples spiked with nevirapine at a concentration range of 1, 3, 5, 8 and 10 µg/ml on different days for 5 days. Calibration curves were created by plotting the peak area ratio of nevirapine to chlorzoxazone, against the spiked concentrations of nevirapine. The curves were analysed by linear regression using the GraphPad® InStat statistical program.

Accuracy was tested at 1, 5 and 10 µg/ml. The test was repeated five times for each sample. Accuracy values were derived from a calibration curve. The results obtained were used to calculate the coefficient of variation using the following formula: (standard deviation/mean) x 100.

Stability of nevirapine in plasma was determined at 5 µg/ml. The sample was stored at room temperature, 4°C and -20°C, and run after 8, 12 and 24 hours and 1 week.

5.6 Application of the method

The method was tested by analysing plasma samples of rats, after oral administration of nevirapine. The details on the animal study and procedures including ethical approval are described in Chapter 6.

5.7 Results

5.7.1 Chromatographic performance

Figures 5.1 a) - 5.1 e) are the representative chromatograms for the standard solutions, blank plasma and spiked plasma. From the standard solutions it was observed that the peaks were well resolved, with nevirapine eluting at 3.176 minutes and the internal standard at 6.873 minutes. The blank plasma showed no interference from plasma. This observation was also clear in the spiked plasma samples. Retention times for plasma spiked with nevirapine and chlorzoxazone were 2.598 and 5.268 minutes respectively. The total run time was 8 minutes.

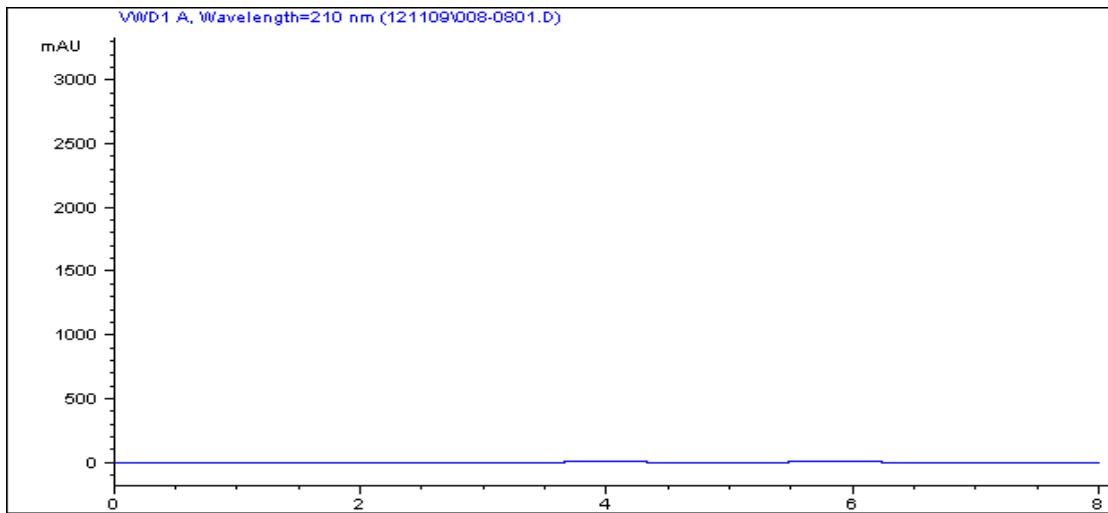


Figure 5.1 a): Chromatogram of mobile phase alone

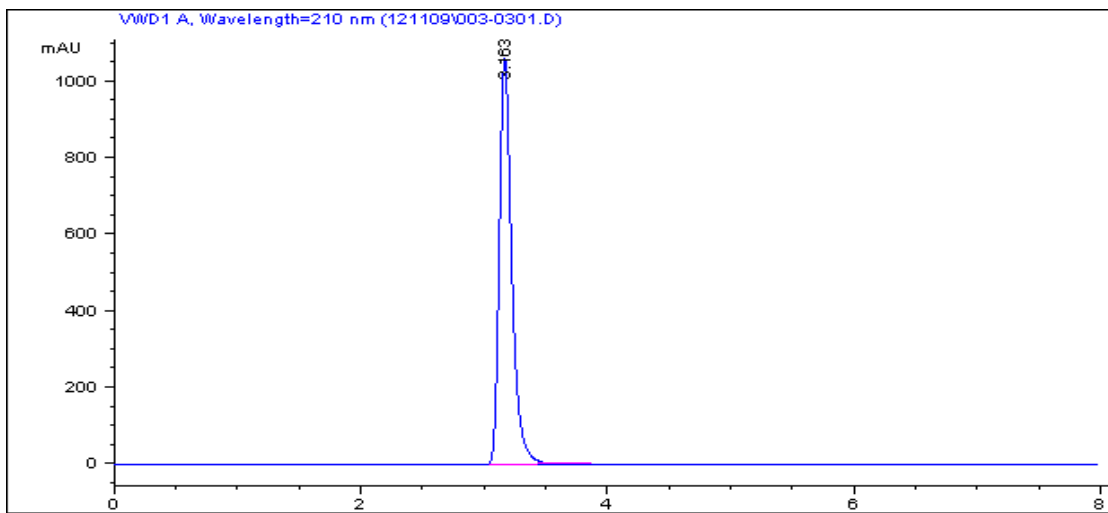


Figure 5.1 b): Chromatogram of mobile phase spiked with nevirapine

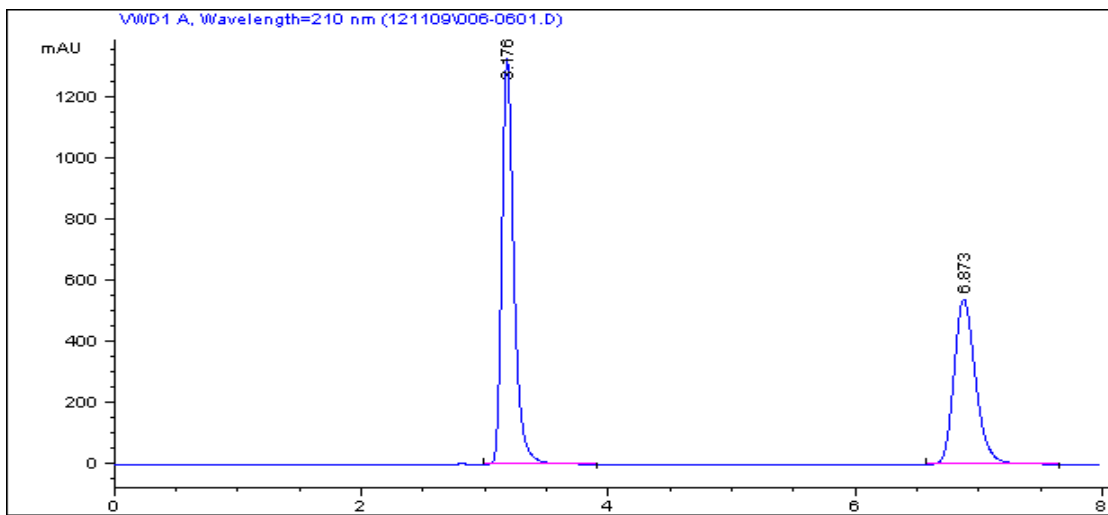


Figure 5.1 c): Chromatogram of mobile phase spiked with nevirapine and internal standard

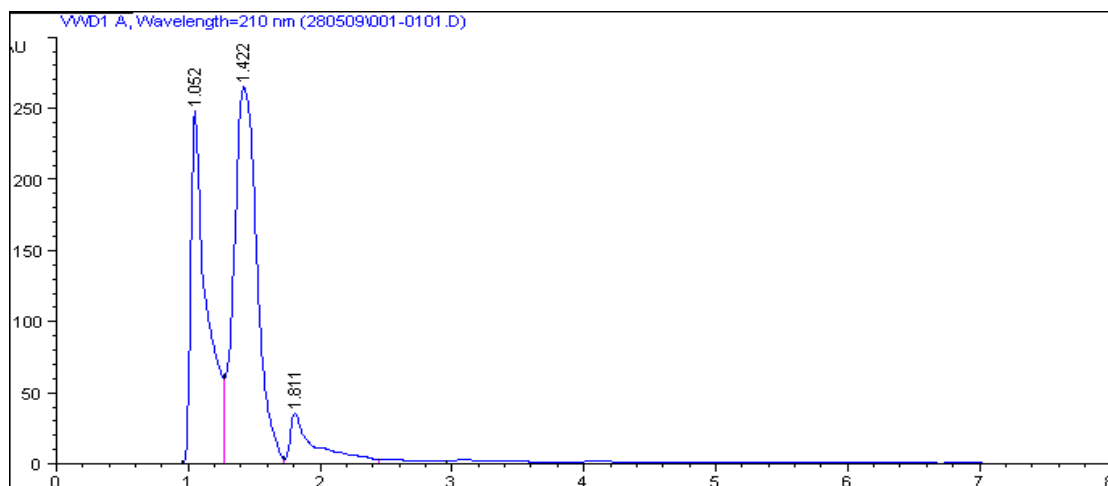


Figure 5.1 d): Chromatogram of a blank plasma sample

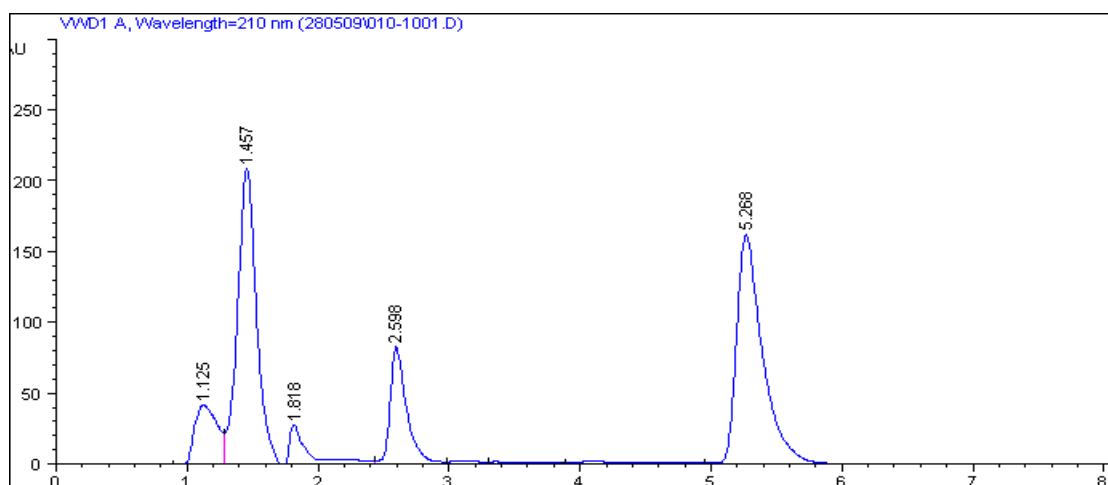


Figure 5.1 e): Chromatogram of a plasma sample spiked with internal standard and 8 µg/ml nevirapine

5.7.2 Calibration curve

The summary data for the calibration over five days is shown in Table 5.1, while the average calibration curve is shown in Figure 5.2 (see Appendix A for individual calibrations). The calibration curve was linear with a regression equation of $y = 0.012x + 0.051$ and correlation coefficient of $r^2 = 0.9985$. The coefficient of variation (CV %) was less than 10%.

Table 5.1: HPLC calibrations for nevirapine over 5 days using ratios of area nevirapine/area internal standard

| Conc. (µg/ml) | Cal. Day 1 | Cal. Day 2 | Cal. Day 3 | Cal. Day 4 | Cal. Day 5 | Mean | SD | CV % |
|---------------|------------|------------|------------|------------|------------|--------------|-------|------|
| 1 | 0.033 | 0.029 | 0.033 | 0.030 | 0.027 | 0.030 | 0.003 | 8.6 |
| 3 | 0.055 | 0.057 | 0.058 | 0.053 | 0.052 | 0.055 | 0.003 | 4.6 |
| 5 | 0.083 | 0.077 | 0.080 | 0.079 | 0.072 | 0.078 | 0.004 | 5.2 |
| 8 | 0.114 | 0.119 | 0.114 | 0.110 | 0.109 | 0.113 | 0.004 | 3.5 |
| 10 | 0.132 | 0.146 | 0.147 | 0.143 | 0.139 | 0.140 | 0.006 | 4.3 |

SD = standard deviation; CV % = coefficient of variation

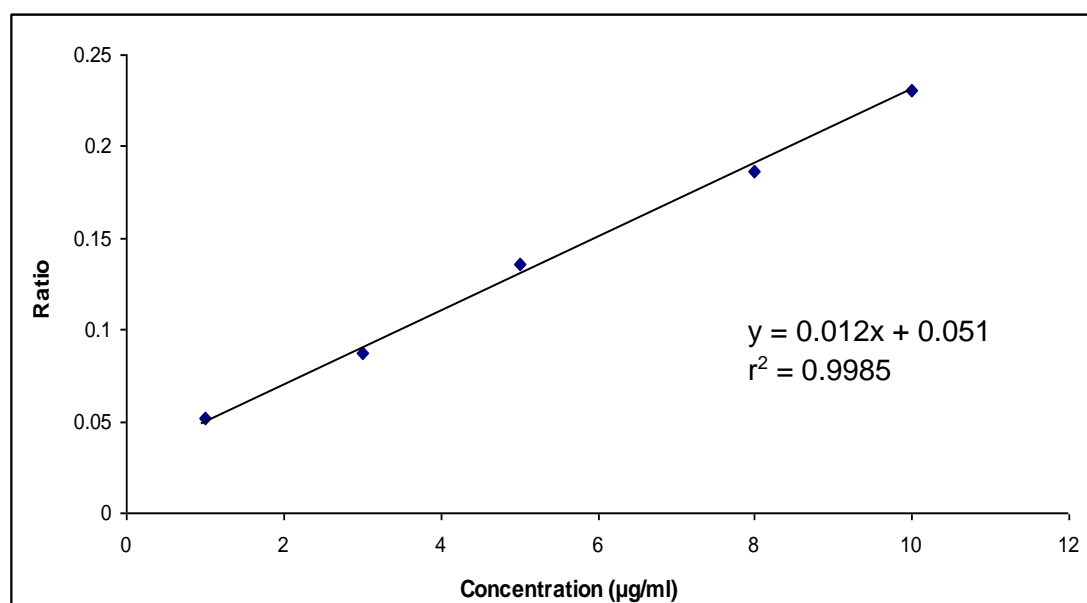


Figure 5.2: 5 Day calibration curve of nevirapine

5.7.3 Accuracy

According to the data in Table 5.2, accuracy was 90%, 101% and 96% at 1, 5 and 10 µg/ml, respectively (view Appendix B for fully detailed accuracy data tables). The CV % was <15 for the 5 and 10 µg/ml samples. For the 1 µg/ml sample the coefficient of variation was slightly higher, owing to the small volume of the sample.

5.7.4 Stability

A variation in nevirapine concentrations was observed over seven days (Table 5.3; Appendix C). It would be advisable to immediately freeze samples when not in use, or just after blood collection.

Table 5.2: Summary of accuracy data of nevirapine in plasma at 1, 5 and 10 µg/ml

| Conc. prepared (n = 5) (µg/ml) | Conc. measured (µg/ml) | Mean accuracy (%) | SD | CV% |
|-----------------------------------|---------------------------|-------------------|-----|------|
| 1 | 0.899 | 90 | 0.2 | 17.7 |
| 5 | 5.030 | 101 | 0.7 | 14.2 |
| 10 | 9.626 | 96 | 0.6 | 5.9 |

SD = standard deviation; CV % = coefficient of variation

Table 5.3: Summary of stability data of 5 µg/ml nevirapine in plasma at room temperature, 4°C and -20°C measured after 8, 12 and 24 hours and 1 week

| Temp. (n = 2) | 8 Hours | | 12 Hours | | 24 Hours | | 1 Week | |
|------------------|-------------------|---------|-------------------|---------|-------------------|---------|-------------------|---------|
| | Conc. measured | % Stab. | Conc. measured | % Stab. | Conc. measured | % Stab. | Conc. measured | % Stab. |
| Room temp. | 6.89 ± 0.3 | 138 | 6.70 ± 0.1 | 134 | 5.74 ± 0.7 | 115 | - | - |
| 4°C | 5.79 ± 0.4 | 116 | 5.77 ± 0.2 | 115 | 5.34 ± 0.1 | 107 | - | - |
| -20°C | 6.32 ± 0.4 | 126 | 6.56 ± 1.7 | 131 | 4.58 ± 0.9 | 92 | 4.30 ± 2.4 | 86 |

5.7.5 Application of the method

Figures 5.3 a) and 5.3 b) show the chromatograms of blank rat plasma, and nevirapine in rat plasma at 6 hours after 200 mg/kg/day administration, respectively. Nevirapine concentration was calculated as 5.67 $\mu\text{g/ml}$.

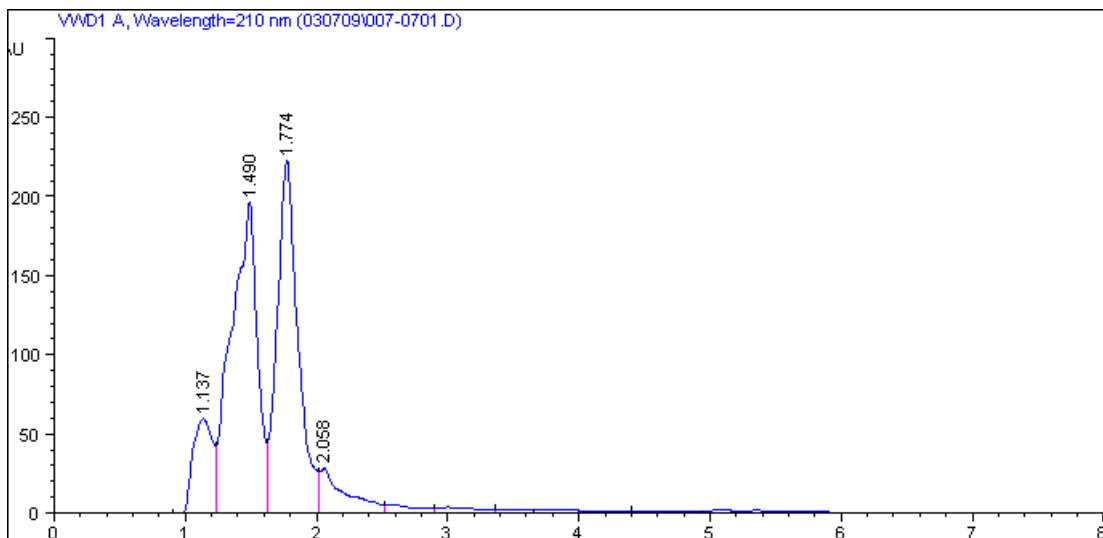


Figure 5.3 a): Chromatogram of blank rat plasma

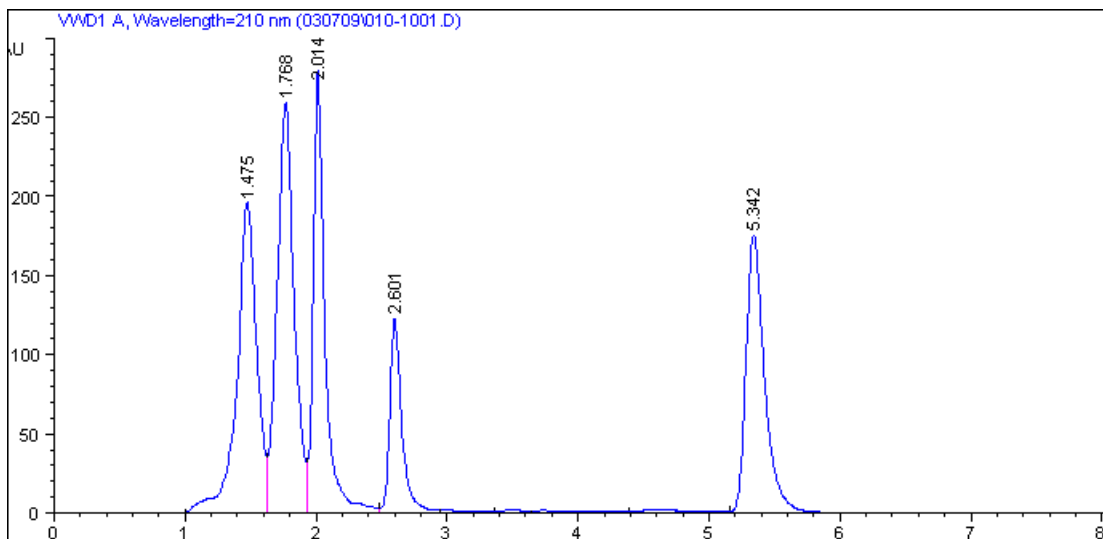


Figure 5.3 b): Chromatogram of nevirapine (5.67 $\mu\text{g/ml}$) in rat plasma at 6 hours after 200 mg/kg/day oral administration

5.8 Comment

An accurate and effective HPLC method for measurement of nevirapine in a small plasma volume (100 µl) was developed. Sharp, symmetrical peaks of nevirapine and the internal standard were observed in the chromatograms produced (Figure 5.1 e). The average calibration curve (Figure 5.2) was linear with a regression equation of $y = 0.012x + 0.051$ and correlation coefficient of $r^2 = 0.9985$; in addition the CV% was $\pm 5.25\%$ (Table 5.1). Accuracy at 1, 5 and 10 µg/ml was 90%, 101% and 96% respectively (Table 5.2), while stability at -20°C was 92% at 24 hours and 86% at 1 week (Table 5.3). The method was used to measure nevirapine concentration in rats (Figure 5.3 b).

The short run time of 8 minutes is advantageous when analysing large sample batches. Care should be taken when performing the solid phase extraction, as it is a small sample volume containing the compounds of interest. Unfortunately nevirapine proved to be unstable, delivering poor stability results. Therefore, it is advisable to analyse nevirapine plasma samples as soon as possible after blood collection. Repeated freeze-thaw cycles should also be avoided. In spite of this shortcoming, the method produced satisfactory results as attention was paid to the time of storage of the samples.

This method will be useful for analysis of nevirapine in patients, particularly in newborns and in small research animals (mice and rats), where most often only a small volume of blood is available.

DETERMINATION OF THE ROLE OF THE IMMUNE SYSTEM IN ACUTE AND CHRONIC NEVIRAPINE INDUCED HEPATOTOXICITY

6.0 Summary

Nevirapine is associated with severe skin and hepatic hypersensitivity reactions that have hampered its use, particularly for HIV prophylaxis. Therefore the role of the immune system in nevirapine induced hepatotoxicity was investigated here. Ethical approval was obtained, and Sprague-Dawley (SD) rats were used. In short, the animal experiment was divided into two phases. In the acute phase, three groups of 10 rats each were administered with bacterial lipopolysaccharide (LPS; 2.9×10^6 E.U./kg, intraperitoneally) or saline (S) followed by oral nevirapine (200 mg/kg) or saline, after which rats were sacrificed at 6 and 24 hours. For the chronic phase, two groups of 15 rats each received daily nevirapine (200 mg/kg), and on days 7, 14 and 21 five rats from each group were administered with either LPS or saline, followed within 5 minutes by that day's nevirapine dose, and were sacrificed 24 hours later. Blood was analysed for ALT, IL-2, IFN- γ , TNF- α , full blood count and nevirapine concentrations. A piece of liver was sent for histopathology testing. Nevirapine caused hepatotoxicity up to seven days and progressively increased IL-2, IFN- γ and TNF- α levels, and lymphocyte count over 21 days. Nevirapine hepatotoxicity was characterised by apoptosis and degeneration changes, while for LPS it was cell swelling, leukostasis and portal inflammation. Co-administration of nevirapine and LPS attenuated nevirapine induced hepatotoxicity, exhibited lower IL-2 and IFN- γ levels, with increased neutrophil and lymphocyte count, and nevirapine concentrations. Nevirapine stimulated the immune system, leading to hepatotoxicity that was prevented by co-administration with LPS, and this implies that manipulation of the immune system may help to prevent nevirapine induced hepatotoxicity.

6.1 Introduction

Nevirapine is liable to hypersensitivity reactions such as skin rash and hepatotoxicity. As cited earlier in the review, involvement of the immune system in nevirapine induced skin rash has already been proven in rats and reported as such (Popovic *et al.*, 2006; Shenton *et al.*, 2003). This, however, has not yet been reported for nevirapine induced hepatotoxicity.

This chapter describes the investigations performed in order to establish whether the immune system played a role in acute and chronic nevirapine induced hepatotoxicity.

6.2 Materials and reagents

6.2.1 Apparatuses

Precision and analytical balances (SPB 52 and SPB 31, Scaltec Instruments, Goettingen, Germany) were used to weigh rats and gram and milligram amounts of reagents and drug standards, respectively. Feeding needles (16 G-3", curved 3mm ball; Poppers and sons, Inc., NY, U.S.A) were used for oral gavage, and a dissection kit (Lasec S.A., Bloemfontein, South Africa) was used to perform rat surgeries. A vortex mixer (Vortex Genie 2, Scientific Industries Inc., Bohemia, NY, U.S.A) and a micro centrifuge (Minispin, Eppendorf, Hamburg, Germany) were used for mixing and quick spinning of the rat plasma samples. Purification of rat plasma samples was performed by solid phase extraction with C₁₈ solid phase extraction cartridges (Sep-Pak[®], Waters Corporation, Milford, MA, U.S.A) along with a solid phase extraction vacuum manifold (Visiprep-DL[™], Bellefonte, PA, U.S.A). The HPLC system (Hewlett Packard model 1100) for nevirapine analysis was equipped with an autosampler (Waldbronn, Germany), isocratic pump (Waldbronn, Germany) and UV detector (Tokyo, Japan). Compounds were separated using a Luna C₁₈ (4.60 x 150 mm) 5 μ analytical column, coupled to a SecurityGuard[™] C₁₈ (4 x 3 mm) guard column (Phenomenex[®], Torrance, CA, U.S.A). Blood samples were centrifuged with a bench centrifuge (5810R, Eppendorf, Hamburg, Germany). ELISAs were performed using a microplate reader (Multiskan Ascent[®], Thermo Electron Corporation, Vantaa, Finland) and microplate washer (93PW, Tecan, Grödig, Austria) equipped with a vacuum pump (KNF Neuberger, Freiburg, Germany).

6.2.2 Materials

Nevirapine as an oral suspension (50 mg/5 ml Viramune[®]) and tablets (200 mg Viramune[®]; Boehringer Ingelheim Pharmaceuticals, Inc., Ridgefield, CT, U.S.A) were purchased from a local pharmacy. Saline solution (Euro-Med Laboratories Inc., Cavite, Philippines) was kindly sponsored by our Toxicology Laboratory. Lipopolysaccharide (from *Escherichia coli*), potassium chloride, chlorzoxazone

and formaldehyde were supplied by Sigma-Aldrich™ (St. Louis, MO, U.S.A). Sodium phosphate monobasic, sodium phosphate dibasic powder, tetraethylammoniumhydroxide (C₈H₂₁NO), orthophosphoric acid (H₃PO₄) and perchloric acid (HClO₄) were obtained from Merck (Darmstadt, Germany). HPLC grade diethyl ether, methanol and acetonitrile were purchased from Honeywell, Burdick and Jackson International Inc. (Muskegon, MI, U.S.A). Liquid nitrogen was obtained from Parexel International (Bloemfontein, South Africa). A rat tumour necrosis factor- α ELISA kit was purchased from eBioscience™ (San Diego, CA, U.S.A). ELISA kits for rat interleukin-2 and rat interferon- γ were supplied by Bender MedSystems (Vienna, Austria). The analytical standard of nevirapine was obtained from Boehringer Ingelheim Pharmaceuticals, Inc. (Ridgefield, CT, U.S.A).

6.2.3 Preparation of special buffers

Neutral buffered formalin was prepared by dissolving sodium phosphate monobasic and sodium dibasic in distilled water. Formaldehyde was added to the phosphate buffer to a final ratio of 10:90 (v/v) in order to achieve a 10% solution. The entire mixture was filtered and the pH set at 6.8.

6.3 Animal care

Ethical approval (ETOVS 06/08) was obtained from the Animal Ethics Committee of the University of the Free State before the animal experiments were started. Sprague-Dawley (SD) rats weighing between 245 – 440 g were used. Animals were kept and treated at the Animal House of the University of the Free State. Here they were fed and looked after by qualified staff and their cages were cleaned once a week. Standard rat food and water was available to the animals *ad libitum*. All animals were inspected for skin lesions and other visible adverse events every day. During the treatment period no physical side-effects or liver injury were observed. All of the rats showed healthy weight gain during each experiment. During oral administration the respective drug was drawn into a syringe and administered to the rats with a feeding needle by gavage. For intraperitoneal administration the respective drug was drawn into a syringe and administered to the rats by direct injection into the peritoneum with a 26G needle.

6.4 Experimental design

6.4.1 Original experimental design

Originally, the animal experiment was divided into two phases, namely, the control phase and test phase. For each phase, rats would be weighed and divided into three groups of 20 animals each. For each group five rats would be studied every 7 days over a 28 day period (Figure 6.1). Nevirapine, lipopolysaccharide (LPS) and saline would be administered as follows for each phase:

6.4.1 a) Control phase

- Control A (Saline): 2 ml saline solution (orally)
- Control B (NVP low): 25 mg/kg/day nevirapine (1 ml, orally)
- Control C (NVP high): 50 mg/kg/day nevirapine (2 ml, orally)

6.4.1 b) Test phase

- Test A (LPS): 2 ml saline (orally) and 2.9×10^6 E.U./kg LPS (1 ml, intraperitoneally)
- Test B (LPS+NVP low): 25 mg/kg/day nevirapine (1 ml, orally) and 2.9×10^6 E.U./kg LPS (1 ml, intraperitoneally)
- Test C (LPS+NVP high): 50 mg/kg/day nevirapine (2 ml, orally) and 2.9×10^6 EU/kg LPS (1 ml, intraperitoneally)

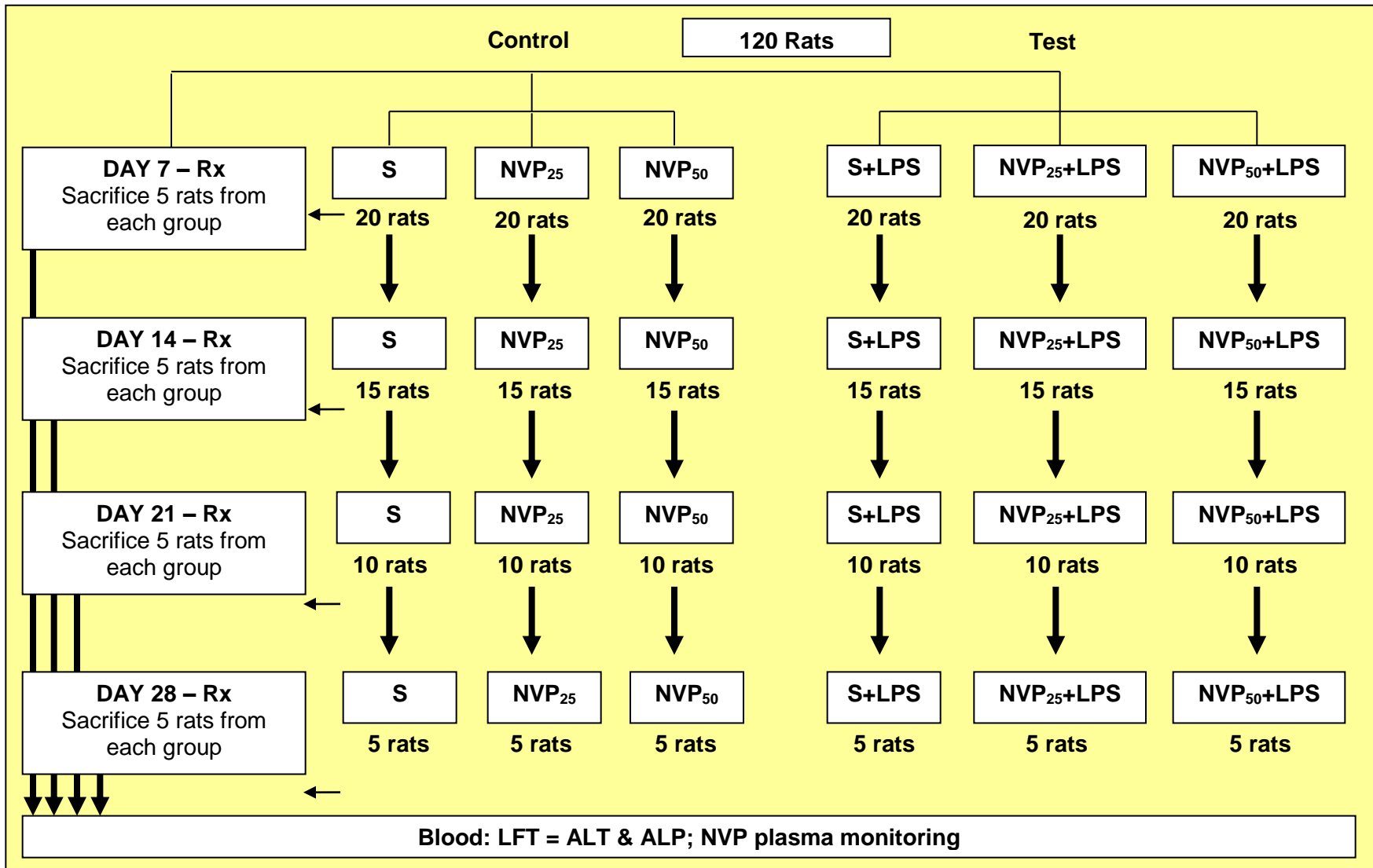


Figure 6.1: A schematic illustration of the original experimental design

6.4.2 Preliminary experiment

6.4.2 a) Methods

i) Dosing schedule

Treatment was started for the control phase groups only (as described in Section 6.4.1 a; Figure 6.1), as 60 rats are a large number of animals to attend to. This decision was made, not only because the treatment was labour intensive, but also to establish whether the selected dosages of nevirapine would be adequate for the remainder of the study.

ii) Surgical procedure and blood collection

Surgery was performed on the day following the last day of dosing. Rats underwent surgery and blood was collected as later described in Section 6.5.1. Blood was collected for liver function tests and plasma monitoring of nevirapine. A liver section was also preserved for histopathology testing.

iii) Analysis of liver function tests and nevirapine plasma levels

Liver function tests were performed and reported by an independent pathology laboratory (Pathcare Veterinary Laboratory, Bloemfontein, South Africa). Plasma levels of nevirapine were analysed by the HPLC assay developed as described in Chapter 5.

iv) Results

During the preliminary experiment, control groups A (saline), B (NVP low) and C (NVP high) showed no physical side-effects or liver injury during the 28 day treatment period. As indicated in Table 6.1, all rats showed healthy weight gain (Appendix D), and it was also observed that there were no significant changes in the liver enzyme levels over time, within and between groups. This observation was most likely due to the dose of nevirapine being too low in both groups B (NVP low) and C (NVP high). These findings were also confirmed by low nevirapine plasma concentrations after HPLC analysis (refer to Appendix H).

Table 6.1: Mean \pm SD values of rat weights, liver function tests and nevirapine plasma concentrations of groups A (saline), B (NVP low) and C (NVP high)

| Group (n = 5) | Weight (g) | | | Liver function tests (units/l) | | | NVP ($\mu\text{g/ml}$) |
|---------------------|--------------|--------------|-------------------------------|--------------------------------|---------------|-----------------|-----------------------------|
| | Before Rx | After Rx | Change | ALP | ALT | AST | |
| A (saline) | | | | | | | |
| 7 Days | 392 \pm 13 | 404 \pm 12 | 12 \pm 4 | 262.5 \pm 11 | 67.8 \pm 24 | 169.3 \pm 107 | - |
| 14 Days | 399 \pm 14 | 419 \pm 21 | 21 \pm 10 | 273.8 \pm 57 | 55.4 \pm 5 | 115.8 \pm 10 | - |
| 21 Days | 376 \pm 24 | 397 \pm 11 | 22 \pm 4 | 285.0 \pm 34 | 57.8 \pm 6 | 120.8 \pm 15 | - |
| 28 Days | 371 \pm 24 | 408 \pm 30 | 37 \pm 8 | 281.8 \pm 35 | 59.0 \pm 1 | 114.4 \pm 6 | - |
| B (NVP low) | | | | | | | |
| 7 Days | 375 \pm 22 | 389 \pm 30 | 15 \pm 2 | 246.8 \pm 40 | 59.6 \pm 6 | 116.6 \pm 17 | 0.59 \pm 1 |
| 14 Days | 371 \pm 13 | 386 \pm 15 | 16 \pm 4 | 246.8 \pm 24 | 56.2 \pm 3 | 157.8 \pm 70 | 0.17 \pm 0 |
| 21 Days | 356 \pm 22 | 376 \pm 17 | 20 \pm 6 | 256.0 \pm 35 | 57.0 \pm 5 | 122.6 \pm 20 | 0.21 \pm 0 |
| 28 Days | 358 \pm 6 | 386 \pm 11 | 28 \pm 7 | 244.8 \pm 42 | 69.2 \pm 12 | 154.8 \pm 50 | 0.30 \pm 0 |
| C (NVP high) | | | | | | | |
| 7 Days | 358 \pm 18 | 368 \pm 21 | 10 \pm 11 | 241.8 \pm 68 | 56.0 \pm 7 | 87.6 \pm 10 | 0.15 \pm 0 |
| 14 Days | 358 \pm 32 | 372 \pm 35 | 14 \pm 6 | 236.4 \pm 33 | 54.8 \pm 7 | 92.2 \pm 13 | 0.03 \pm 0 |
| 21 Days | 349 \pm 10 | 374 \pm 11 | 25 \pm 4 | 231.4 \pm 31 | 55.4 \pm 2 | 105.2 \pm 30 | 0.04 \pm 0 |
| 28 Days | 364 \pm 19 | 395 \pm 22 | 31 \pm 13 | 255.8 \pm 49 | 56.0 \pm 5 | 99.4 \pm 10 | 0.23 \pm 0 |

Rx = treatment; **ALP** = alkaline phosphatase (56.8 – 128 u/l); **ALT** = alanine aminotransferase (17.5 – 30.2 u/l); **AST** = aspartate aminotransferase (45.7 – 80.8 u/l; Johnson-Delaney, 1996); **NVP** = nevirapine

v) Comment

Nevirapine plasma concentrations were unfortunately too low to be fully integrated by the HPLC assay, indicating that the selected nevirapine dosages were inadequate to perform the remainder of the test phase. In addition, no liver injury was observed, further indicating that the nevirapine dosages were too low. The decision was made not to have these three groups analysed for histopathology and cytokine levels.

It was concluded that the experimental design and nevirapine dose would have to be revised and adjusted in order to achieve the ideal situation in which liver injury and hepatotoxicity would be induced.

6.4.3 Revised experimental design

It was decided to increase the dose of nevirapine to 200 mg/kg/day. Of note, nevirapine induces its own metabolising enzymes, CYP2B6 and CYP3A4, leading to increased metabolism of nevirapine itself (Walubo *et al.*, 2006). Furthermore, the experiment was divided into an acute and a chronic phase.

6.4.4 Ultimate experimental design

6.4.4 a) Acute phase

Rats were weighed and divided into three groups of 10 rats each, namely: group D (S+NVP), group E (LPS+S), and group F (LPS+NVP). For each group five rats were studied at 6 hours after drug administration and the remaining five were studied at 24 hours. Lipopolysaccharide (LPS, measured in endotoxin units), nevirapine and saline solution were administered as follows (Figure 6.2):

- Group D: saline (1 ml, intraperitoneally) and 200 mg/kg nevirapine (2 ml, orally)
- Group E: 2.9×10^6 E.U./kg LPS (1 ml, intraperitoneally) and saline (2 ml, orally)
- Group F: 2.9×10^6 E.U./kg LPS (1 ml, intraperitoneally) and 200 mg/kg nevirapine (2 ml, orally)

The dose of LPS was based on a study performed by Deng *et al.* (2006), and that of nevirapine by Shenton *et al.* (2003).

6.4.4 b) Chronic phase

Rats were weighed and divided into two groups of 15 rats each, namely group G (S+NVP) and group H (LPS+NVP). For each group 5 rats were studied per 7 days over a 21 day period. Both groups received nevirapine orally for 7, 14 and 21 days respectively. On days 7, 14 and 21 group G received saline solution, and group H LPS intraperitoneally. The rats were sacrificed 24 hours after administration of saline or LPS (Deng *et al.*, 2006). Groups G and H were dosed as follows (Figure 6.3):

- Group G: 200 mg/kg/day nevirapine (2 ml, orally) and saline (1 ml, intraperitoneally)
- Group H: 200 mg/kg/day nevirapine (2 ml, orally) and 2.9×10^6 E.U./kg LPS (1 ml, intraperitoneally)

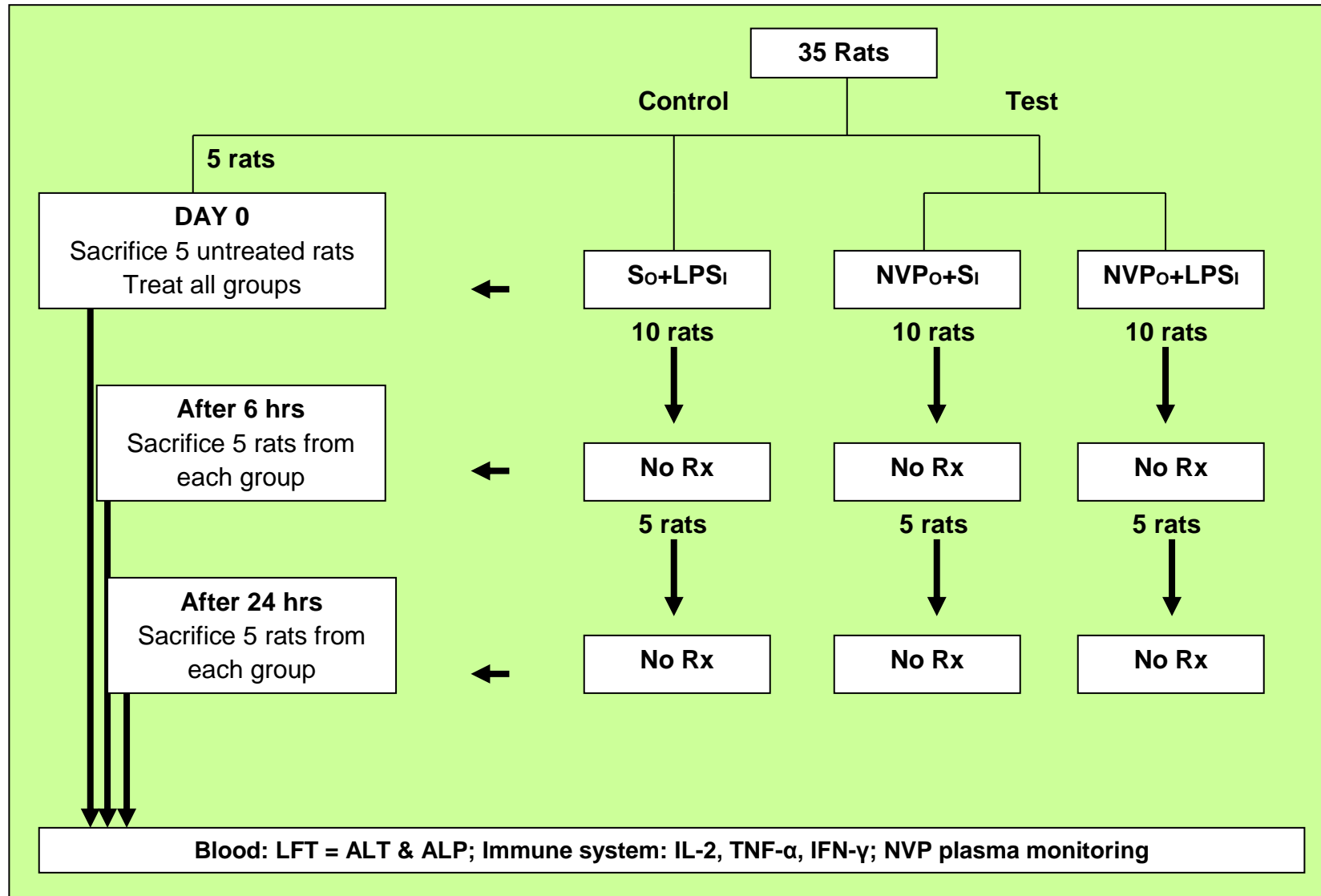


Figure 6.2: A schematic illustration of the experimental design for the acute phase

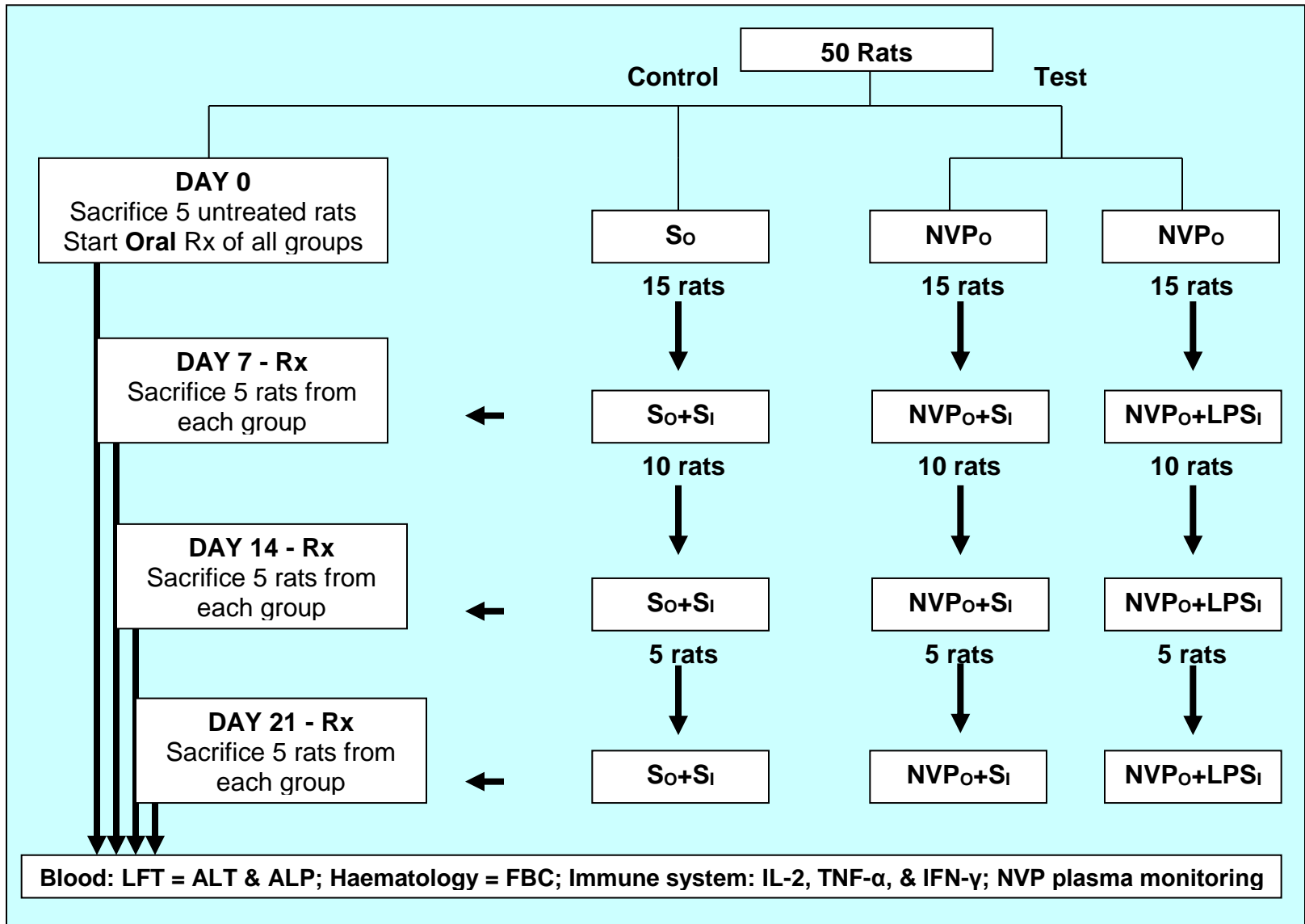


Figure 6.3: A schematic illustration of the experimental design for the chronic phase

6.5 Procedures

6.5.1 Surgical procedure and blood collection

All rats were sacrificed on the day following the last day of dosing, except in the acute phase where rats were sacrificed at 6 and 24 hours after drug administration. The rats were anaesthetised with diethyl ether, and blood was drawn by direct cardiac puncture (Figure 6.4). This procedure differs from the originally proposed method of blood collection via the abdominal vein. It was found that, during cardiac puncture, the rats did not die as quickly as when blood was drawn from the abdominal vein. It was also a faster and cleaner method of blood collection. Thereafter the abdomen was opened and the liver exposed. A liver section was cut and stored in 10% formalin. The remainder of the liver was excised, removed and washed in a 1.5% potassium chloride solution, frozen with liquid nitrogen and stored at -85°C. The rats were sacrificed by exsanguination whilst still under diethyl ether anaesthesia. Blood was collected in yellow top serum separator tubes for liver function tests, green top lithium heparin tubes for plasma, red top serum separator tubes for serum, and purple top EDTA tubes for full blood count. The plasma and serum tubes were centrifuged and aliquots stored at -20°C until analysis. Blood for liver function tests and full blood count was sent off to an independent laboratory for analysis (Section 6.5.2).



Figure 6.4: An illustration of direct heart puncture for blood collection under ether anaesthesia

6.5.2 Analysis of liver function tests, full blood count and histopathology

All liver function tests were conducted and reported by Pathcare Veterinary Laboratory (Bloemfontein, South Africa). The following serum transaminase enzymes levels were measured on the day of surgery: alanine aminotransferase (ALT), aspartate aminotransferase (AST) and alkaline phosphatase (ALP).

Full blood count was also determined and reported by Pathcare Veterinary Laboratories. It was only requested for groups G (S+NVP) and H (LPS+NVP) after 21 days of treatment to establish the presence of possible infection, as LPS is derived from the bacteria *Escherichia coli*. The following parameters were analysed and reported: red blood cells, white blood cells and platelets.

Histopathological diagnosis of the rat livers was performed and reported by an independent veterinary pathologist (Golden Vetpath, Idexx Laboratories, Johannesburg, South Africa). As mentioned in Section 6.5.1, liver sections were cut from the rat livers and placed in 10% neutral buffered formalin until analysis.

6.5.3 Analysis of cytokines by enzyme-linked immunosorbent assay

An enzyme-linked immunosorbent assay (ELISA) was used for the quantitative detection of rat IL-2, IFN- γ and TNF- α in rat serum. All assays were performed in a 96 well microplate as illustrated in Figure 6.5.

| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
|---|--------------------|--------------------|---------------|---------------|------------------|------------------|---------------|---------------|----------------|----------------|----------------|----------------|
| A | Blank | Blank | GrE 6hRat1 | GrE 6hRat1 | GrE 24hRat1 | GrE 24hRat1 | GrG D7Rat1 | GrG D7Rat1 | GrG D14Rat1 | GrG D14Rat1 | GrG D21Rat1 | GrG D21Rat1 |
| B | Std 1 31.3pg/ml | Std 1 31.3pg/ml | GrE 6hRat2 | GrE 6hRat2 | GrE 24hRat2 | GrE 24hRat2 | GrG D7Rat2 | GrG D7Rat2 | GrG D14Rat2 | GrG D14Rat2 | GrG D21Rat2 | GrG D21Rat2 |
| C | Std 2 62.5pg/ml | Std 2 62.5pg/ml | GrE 6hRat3 | GrE 6hRat3 | GrE24 24hRat3 | GrE24 24hRat3 | GrG D7Rat3 | GrG D7Rat3 | GrG D14Rat3 | GrG D14Rat3 | GrG D21Rat3 | GrG D21Rat3 |
| D | Std 3 125pg/ml | Std 3 125pg/ml | GrF 6hRat1 | GrF 6hRat1 | GrF 24hRat1 | GrF 24hRat1 | GrG D7Rat4 | GrG D7Rat4 | GrH D14Rat1 | GrH D14Rat1 | GrH D21Rat1 | GrH D21Rat1 |
| E | Std 4 250pg/ml | Std 4 250pg/ml | GrF 6hRat2 | GrF 6hRat2 | GrF 24hRat2 | GrF 24hRat2 | GrH D7Rat1 | GrH D7Rat1 | GrH D14Rat2 | GrH D14Rat2 | GrH D21Rat2 | GrH D21Rat2 |
| F | Std 5 500pg/ml | Std 5 500pg/ml | GrF 6hRat3 | GrF 6hRat3 | GrF 24hRat3 | GrF 24hRat3 | GrH D7Rat2 | GrH D7Rat2 | GrH D14Rat3 | GrH D14Rat3 | GrH D21Rat3 | GrH D21Rat3 |
| G | Std 6 1000pg/ml | Std 6 1000pg/ml | GrF 6hRat4 | GrF 6hRat4 | GrF 24hRat4 | GrF 24hRat4 | GrH D7Rat3 | GrH D7Rat3 | GrH D14Rat4 | GrH D14Rat4 | GrH D21Rat4 | GrH D21Rat4 |
| H | Std 7 2000pg/ml | Std 7 2000pg/ml | GrF 6hRat5 | GrF 6hRat5 | GrF 24hRat5 | GrF 24hRat5 | GrH D7Rat4 | GrH D7Rat4 | GrH D14Rat5 | GrH D14Rat5 | GrH D21Rat5 | GrH D21Rat5 |

Figure 6.5: An illustration of a 96 well microplate layout for measuring IFN- γ

The concentration range of each standard curve, time of incubation and washing steps differed from kit to kit. In general, the assay was performed according to the manufacturer's instruction as follows:

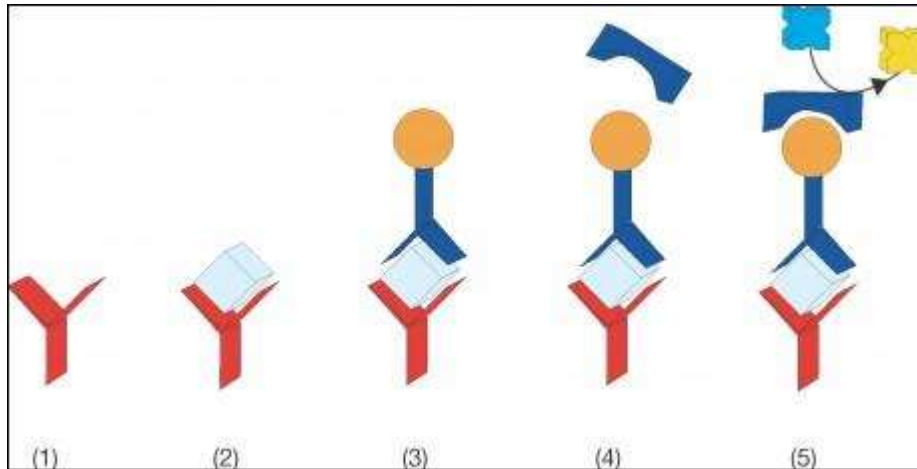


Figure 6.6: An illustration of the principles of ELISA (Available from: <http://www.bendermedsystems.com/elisa--22>)

1. The 96 well ELISA microplate was already coated with a capture antibody, specific to the cytokine to be analysed.
2. The sample was added and the respective antigen present in the sample bonded to the capture antibody.
3. A biotin-conjugated secondary detection antibody was added, that bonded to the antigen captured by the first antibody.
4. Streptavidin-horseradish peroxidase was added and bonded to the biotin-conjugated detection antibody.
5. A coloured product was formed in proportion to the amount of antigen present, and the reaction was stopped by the addition of acid after which absorption was measured at 450 nm with a microplate reader (Bender MedSystems, 2007).

A standard curve was prepared from rat cytokine standard dilutions and the rat cytokine sample concentration was derived from the standard curve in pg/ml.

For IL-2 the standard curve concentration range was 63, 125, 250, 500, 1000 and 2000 pg/ml. IFN- γ and TNF- α had a standard concentration range of 31.3, 62.5, 125, 250, 500, 1000 and 2000 pg/ml.

All samples were analysed in duplicate.

6.5.4 Analysis of nevirapine in rat plasma by the developed HPLC assay

Nevirapine levels in rat plasma were monitored using the HPLC assay developed and described in Chapter 5. A standard curve was generated from 5 known nevirapine calibration standards, from which nevirapine concentrations in rat plasma were derived.

6.6 Statistical analysis

Data were analysed by non-parametric methods using the GraphPad InStat statistical program. Accordingly, parameters were reported as mean and standard deviation (SD), and the Kruskal-Wallis Test, as well as the Dunn's Multiple Comparisons Test, was used for data comparison with the level of significance set at $p < 0.05$.

6.7 Results

6.7.1 Direct observations

During treatment there were no side-effects except that on the day of sacrifice, *i.e.*, 24 hours after administration of LPS or saline. The animals administered with LPS exhibited goose-flesh (raised hair) and, on inspection of the liver during surgery, there were goose bumps on their liver surface. Unfortunately, temperature was not taken, but this was interpreted as due to the immune reaction akin to serum-sickness.

6.7.2 Acute phase

6.7.2 a) Hepatotoxicity

Table 6.2 shows the weights and liver function tests of animals in the acute phase, while Figures 6.7 a – 6.7 g show the representative photographs of the liver histopathology and Table 6.3 is a tally of the main pathological lesions as indicated in the histopathology reports.

i) Liver function tests

In Table 6.2 the liver function test results (ALT and AST) for all test animals were not different from those of the control (untreated group) at 6 and 24 hours ($P > 0.05$; Appendix I). Although the ALT and AST values are higher than quoted in the literature (Johnson-Delaney, 1996), this is not a sign of hepatotoxicity in our setting where high values have been consistently observed (ALT = 63 – 118 μ l; Walubo *et al.*, 2004 and 2006). Even then, there were differences, though not significant, in ALT levels at 6 and 24 hours within the respective test groups (Appendix I).

Surprisingly, the ALT observations did not correlate with the histopathology changes in Figures 6.7 a – 6.7 g. Here, there was no hepatotoxicity in the control group (Figure 6.7 a), while there were hepatotoxicity changes in the test groups (Figure 6.7 b – 6.7 g). This implies that the hepatotoxicity was subclinical, *i.e.*,

one could not use clinical parameters to detect hepatotoxicity as there were no signs and symptoms. In fact, diagnosis of hepatotoxicity by ALT is usually possible when ALT is more than three times its normal value. Therefore, from here on the histopathology results were used as the indicators of hepatotoxicity.

ii) S+NVP group

From the histopathology results, the saline+nevirapine (S+NVP) group exhibited hepatotoxicity at both 6 (Figure 6.7 b) and 24 hours (Figure 6.7 c). From the report, nevirapine hepatotoxicity at 6 hours was characterised by mild cloudy swelling and degenerative changes with granular appearance of hepatocytes, increased apoptosis and diffuse mild hepatocellular swelling (Section 6.7.2 b ii, pg. 72), while at 24 hours, there was hepatocellular vacuolar degeneration, apoptosis and dissociated liver parenchymal cells (Section 6.7.2 b iii, pg. 72). These lesions are also summarised in the tally table (Table 6.3).

iii) LPS+S group

Likewise, the lipopolysaccharide+saline (LPS+S) group exhibited hepatotoxicity at both 6 (Figure 6.7 d) and 24 hours (Figure 6.7 e). From the report, LPS hepatotoxicity at 6 hours was characterised by hypertrophic parenchymal cells leading to narrow sinusoids (Section 6.7.2 b iv, pg. 73), while at 24 hours, there were diffuse vacuolar changes, irregular swollen cytoplasm leading to prominent encroachment of the sinusoids, and apoptosis (Section 6.7.2 b v, pg. 73; Table 6.3).

iv) LPS+NVP group

Surprisingly, the histology results were relatively normal at 6 (Figure 6.7 f) and 24 hours (Figure 6.7 g) after the co-administration of lipopolysaccharide+nevirapine (LPS+NVP). From the report, liver parenchyma appeared undisturbed at 6 hours (Section 6.7.2 b vi, pg. 73), and at 24 hours, hepatic parenchymal cells and portal tracts appeared morphologically normal (Section 6.7.2 b vii, pg. 74; Table 7.3).

In effect, the co-administration of LPS and nevirapine prevented the hepatotoxic effects of both drugs.

v) Localisation of pathological lesions

In the tally of main pathological lesions (Table 6.3) it was observed that LPS hepatotoxicity was mainly extracellular and generalised, while that of nevirapine was intracellular and affected some cells more than others.

Table 6.2: Mean \pm SD values of rat weights and liver function test results of untreated rats and groups D (S+NVP), E (LPS+S) and F (LPS+NVP) at 6 and 24 hours after dosing

| Group (n = 5) | Weight (g) | Liver function (units/l) | | |
|--------------------------|-----------------------|---------------------------------|---------------|-----------------|
| | | ALP | ALT | AST |
| Untreated | | | | |
| 0 Hours | 392 \pm 13 | 262.5 \pm 11 | 67.8 \pm 24 | 169.3 \pm 107 |
| D (S+NVP) | | | | |
| 6 Hours | 372 \pm 26 | 268.0 \pm 49 | 53.2 \pm 6 | 31.4 \pm 65 |
| 24 Hours | 367 \pm 32 | 271.6 \pm 38 | 67.2 \pm 8 | 135.0 \pm 28 |
| E (LPS+S) | | | | |
| 6 Hours | 302 \pm 22 | 214.2 \pm 32 | 83.2 \pm 28 | 161.0 \pm 23 |
| 24 Hours | 286 \pm 20 | 274.8 \pm 35 | 30.4 \pm 3 | 131.4 \pm 57 |
| F (LPS+NVP) | | | | |
| 6 Hours | 379 \pm 18 | 175.6 \pm 32 | 61.0 \pm 16 | 134.2 \pm 57 |
| 24 Hours | 385 \pm 8 | 205.8 \pm 24 | 31.0 \pm 8 | 106.6 \pm 20 |

ALP = alkaline phosphatase; **ALT** = alanine aminotransferase); **AST** = aspartate aminotransferase; **S** = saline; **NVP** = nevirapine; **LPS** = lipopolysaccharide

6.7.2 b) *Histopathology photographs and reports*

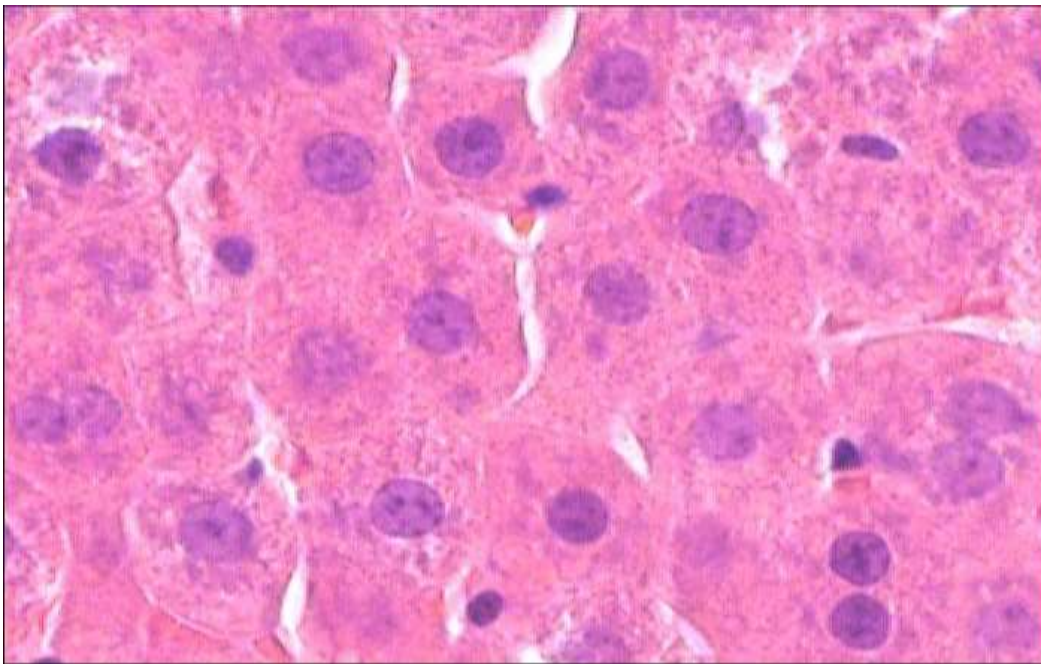


Figure 6.7 a): Liver section from an untreated rat at 0 hours showing no lesions and necrosis, and normal portal tracts with no inflammation (Section 6.7.2 b i, pg. 72)

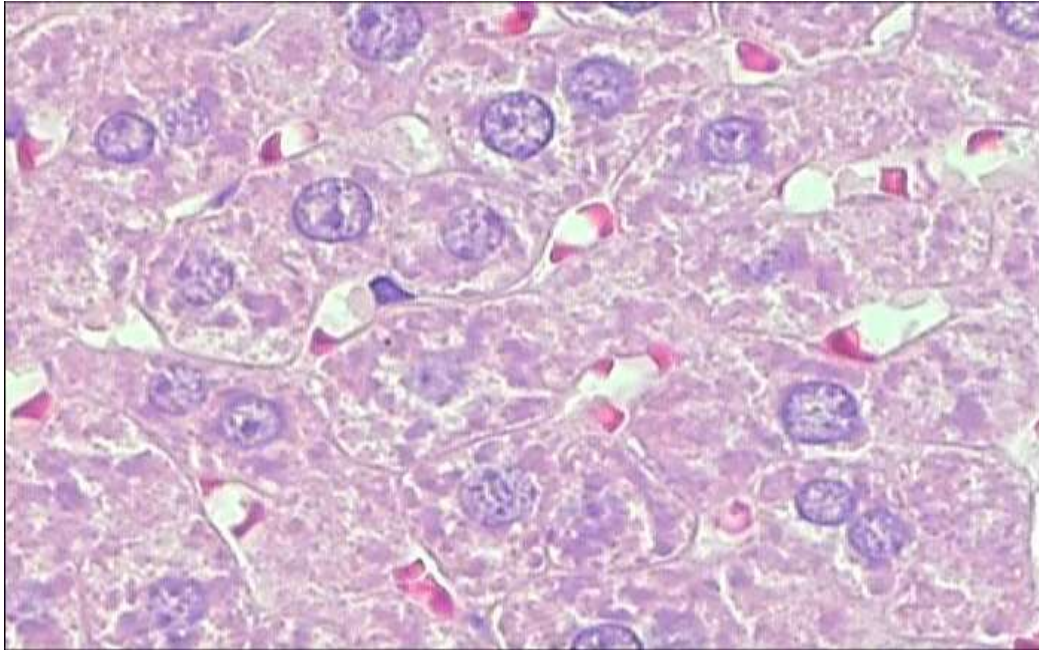


Figure 6.7 b): Liver section from the S+NVP group at 6 hours after dosing, showing mild cloudy swelling and degenerative changes with granular appearance of hepatocytes, increased apoptosis and diffuse mild hepatocellular swelling (Section 6.7.2 b ii, pg. 72)

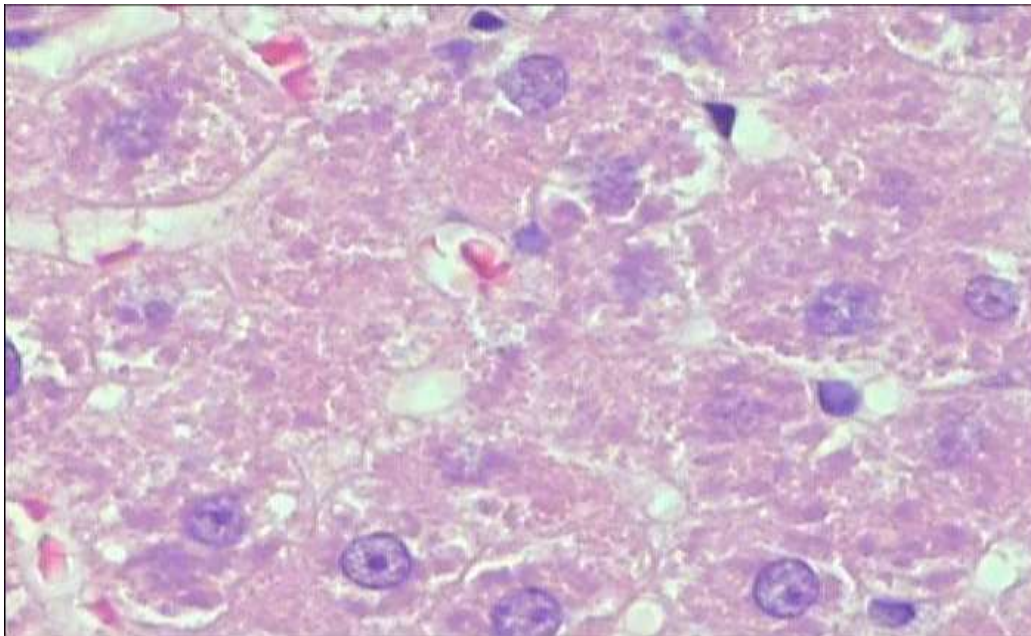


Figure 6.7 c): Liver section from the S+NVP group at 24 hours after dosing, showing hepatocellular vacuolar degeneration, apoptosis and dissociated liver parenchymal cells (Section 6.7.2 b iii, pg. 72)

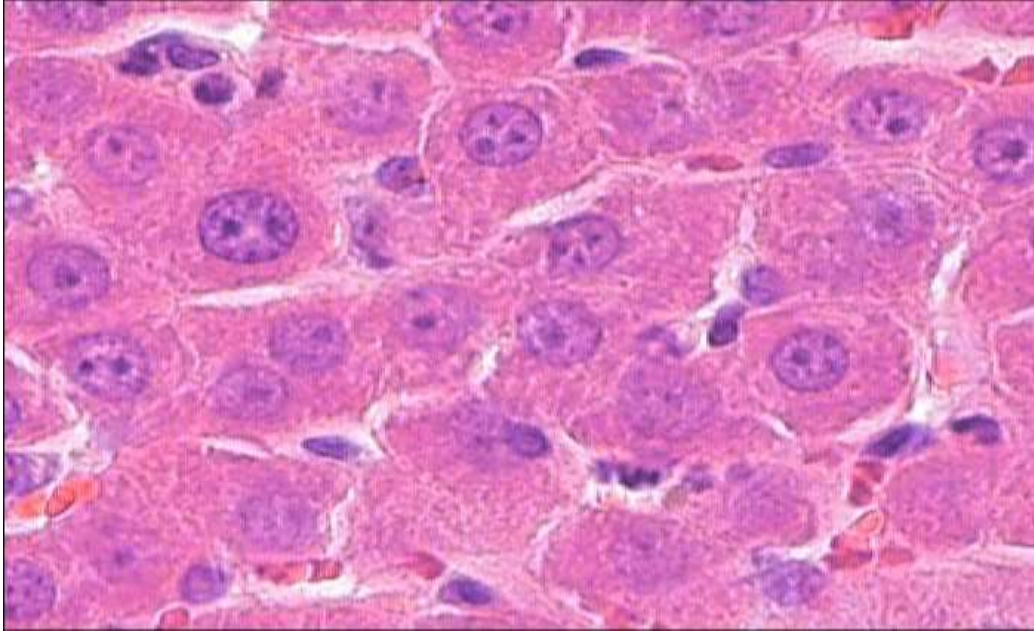


Figure 6.7 d): Liver section from the LPS+S group at 6 hours after dosing, showing hypertrophic parenchymal cells leading to narrow sinusoids (Section 6.7.2 b iv, pg. 73)

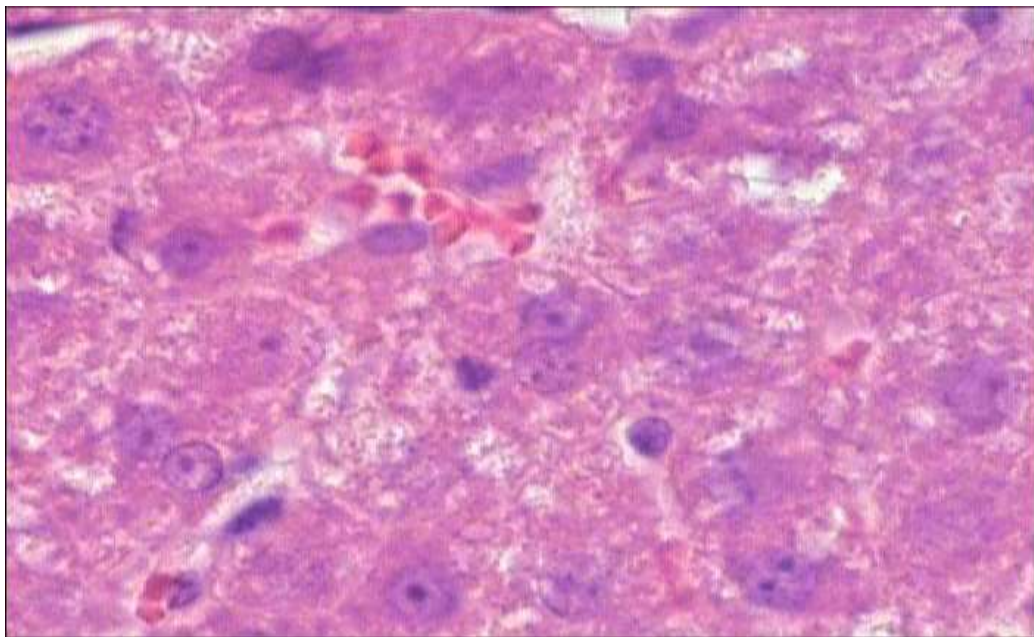


Figure 6.7 e): Liver section from the LPS+S group at 24 hours after dosing, showing diffuse vacuolar changes, irregular swollen cytoplasm leading to prominent encroachment of the sinusoids, and apoptosis (Section 6.7.2 b v, pg. 73)

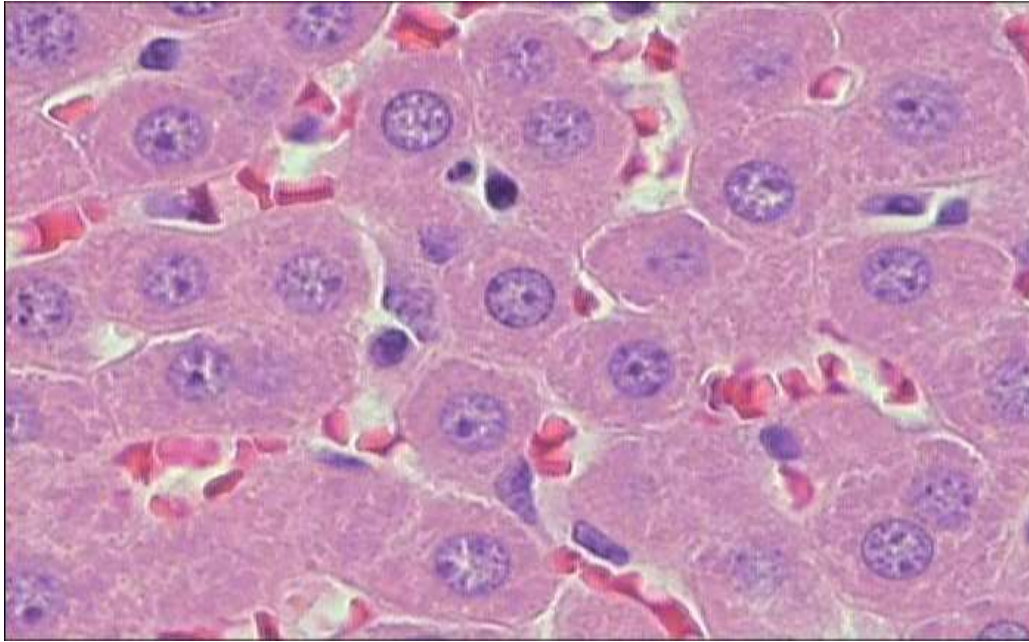


Figure 6.7 f): Liver section from the LPS+NVP group at 6 hours after dosing, showing undisturbed liver parenchyma (Section 6.7.2 b vi, pg. 73)

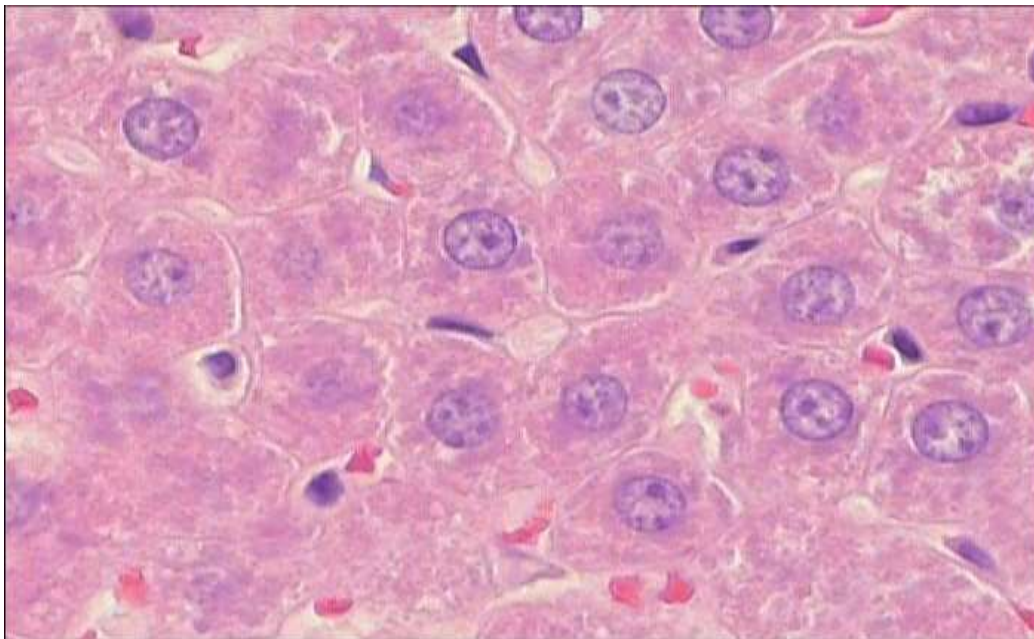


Figure 6.7g): Liver section from the LPS+NVP group at 24 hours after dosing, showing morphologically normal hepatic parenchymal cells and portal tracts (Section 6.7.2 b vii, pg. 74)

Figures 6.7 a – 6.7 g: Histopathology reports

Liver sections for histopathology (Figures 6.7 a – g) were selected and the following diagnosis and remarks were made by the veterinary pathologist.

i) Figure 6.7 a: Liver section from an untreated rat at 0 hours

A representative photograph of a rat liver from an untreated rat. The corresponding pathology report: *“No lesions are observed in the liver parenchyma in the Haematoxylin and Eosin stained section. The portal tracts are normal without any inflammation visible. There is no hepatic parenchymal degeneration or necrosis detected.”*

ii) Figure 6.7 b: Liver section from the S+NVP group at 6 hours after dosing

A representative photograph of a rat liver from group D, dosed with saline and nevirapine at 6 hours after administration. The corresponding pathology report: *“The microscopical evaluation shows mild cloudy swelling and degenerative changes with granular appearance of the cytoplasm with hepatocytes, and in some foci there appears to be increased apoptosis. Diffuse mild hepatocellular swelling could also be demonstrated. The portal tracts appear morphologically normal. There is no bile stasis or any inflammation detected.”*

iii) Figure 6.7 c: Liver section from the S+NVP group at 24 hours after dosing

A representative photograph of a rat liver from group D, dosed with saline and nevirapine at 24 hours after administration. The corresponding pathology report: *“The liver section shows a mild degree of hepatocellular vacuolar degeneration (vacuolar hepatopathy) as well as some foci of apoptosis and dissociated liver parenchymal cells. The portal tracts appear normal without any inflammation visible.”*

iv) Figure 6.7 d: Liver section from the LPS+S group at 6 hours after dosing

A representative photograph of a rat liver from group E, dosed with LPS and saline at 6 hours after administration. The corresponding pathology report:

“In the liver section the parenchymal cells appear (homogenously swollen/hypertrophic) similar to that recorded in LPS-S 1.1 (the hepatic parenchymal cells appear to be swollen with narrow sinusoids present, but no degenerative changes could be confirmed within the cytoplasm of the liver) with minimal intrahepatic leukostasis detected. The portal tracts show minimal inflammation.”

v) Figure 6.7 e: Liver section from the LPS+S group at 24 hours after dosing

A representative photograph of a rat liver from group E, dosed with LPS and saline at 6 hours after administration. The corresponding pathology report:

“In the liver section that was harvested by necropsy 24 hours after intraperitoneal drug administration the liver parenchymal cells revealed diffuse vacuolar changes and irregular swollen cytoplasm with prominent encroachment of the sinusoids due to the cell swelling. Apoptosis of the hepatocytes could be detected. There is mild portal inflammation present, but no inflammation within the hepatic parenchyma.”

vi) Figure 6.7 f: Liver section from the LPS+NVP group at 6 hours after dosing

A representative photograph of a rat liver from group F, dosed with LPS and nevirapine at 6 hours after administration. The corresponding pathology report:

“The liver parenchyma appears undisturbed with no necrosis, excessive apoptosis or any inflammation visible. The portal tracts are morphologically normal. A normal population of Kupffer cells could be detected lining the hepatic sinusoids.”

vii) Figure 6.7 g: Liver section from the LPS+NVP group at 24 hours after dosing

A representative photograph of a rat liver from group F, dosed with LPS and nevirapine at 24 hours after administration. The corresponding pathology report:

“The hepatic parenchymal cells as well as the Kupffer cells lining the sinusoids appear morphologically normal without excessive degeneration or apoptosis visible. The portal tracts are morphologically normal. There is no inflammation or bile stasis visible.”

Table 6.3: Tally of main pathology lesions in rat livers of groups D (S+NVP), E (LPS+S) and F (LPS+NVP) at 6 and 24 hours after dosing

| Pathology | S+NVP | | LPS+S | | LPS+NVP | | Lesion localisation |
|----------------------------------|---------|----------|---------|----------|---------|----------|------------------------------|
| | 6 hours | 24 hours | 6 hours | 24 hours | 6 hours | 24 hours | |
| Swollen cells | + | 0 | ++ | +++ | 0 | 0 | Cellular changes |
| Narrow sinusoids | 0 | 0 | ++ | +++ | 0 | 0 | |
| Leukostasis | 0 | 0 | + | + | 0 | 0 | |
| Portal tract inflammation | 0 | 0 | + | + | 0 | 0 | |
| Parenchyma inflammation | 0 | 0 | 0 | 0 | 0 | 0 | |
| Vacuolar changes | 0 | + | 0 | ++ | 0 | 0 | Intracellular changes |
| Apoptosis | ++ | +++ | 0 | ++ | 0 | + | |
| Degenerative changes | + | ++ | 0 | 0 | 0 | + | |
| Granular appearance | + | 0 | 0 | 0 | 0 | 0 | |
| Dissociated liver cells | 0 | ++ | 0 | 0 | 0 | 0 | |

S = saline; NVP = nevirapine; LPS = lipopolysaccharide

6.7.2 c) Immune response

i) Cytokines

Table 6.5 shows the changes (mean \pm SD) of cytokine profiles, while Figures 6.8 a – 6.8 c are the mean versus time plots of the cytokines. Administration of LPS+S caused a profound increase in IL-2 at 6 and 24 hours, while administration of S+NVP and LPS+NVP led to moderate increase in IL-2 at 6 hours (Figure 6.8 a; Table 6.4). Even then, IL-2 levels were higher in the LPS+NVP group than in the S+NVP group, implying that LPS (not nevirapine) was responsible for the increased IL-2 in the acute phase. There was no change in IFN- γ (Figure 6.8 b; Table 6.4) but S+NVP led to increased concentrations of TNF- α , while the co-administration of LPS and nevirapine blunted this response after 6 hours (Figure 6.8 c; Table 6.4).

Table 6.4: Mean \pm SD values of serum cytokine levels of IL-2, IFN- γ and TNF- α of untreated rats and groups D (S+NVP), E (LPS+S) and F (LPS+NVP) at 6 and 24 hours after dosing

| Group (n = 5) | IL-2 | IFN- γ | TNF- α |
|--------------------|--------------------|-------------------|----------------------|
| Untreated | | | |
| 0 Hours | 238.83 \pm 18.9 | 138.65 \pm 8.3 | 103.43 \pm 44.2 |
| D (S+NVP) | | | |
| 6 Hours | 386.02 \pm 33.4 | 158.72 \pm 74.4 | 874.09 \pm 245.5 |
| 24 Hours | 368.92 \pm 16.6 | 114.09 \pm 30.6 | 3600.63 \pm 232.5 |
| E (LPS+S) | | | |
| 6 Hours | 317.36 \pm 64.6 | 160.23 \pm 30.1 | 56.23 \pm 42.1 |
| 24 Hours | 517.93 \pm 152.3 | 130.50 \pm 19.5 | 41.64 \pm 41.8 |
| F (LPS+NVP) | | | |
| 6 Hours | 421.63 \pm 65.7 | 140.71 \pm 70.1 | 1650.19 \pm 1157.3 |
| 24 Hours | 398.55 \pm 42.9 | 123.33 \pm 19.4 | 646.09 \pm 360.9 |

IL-2 = interleukin-2; IFN- γ = interferon- γ ; TNF- α = tumour necrosis factor- α ; S = saline; NVP = nevirapine; LPS = lipopolysaccharide

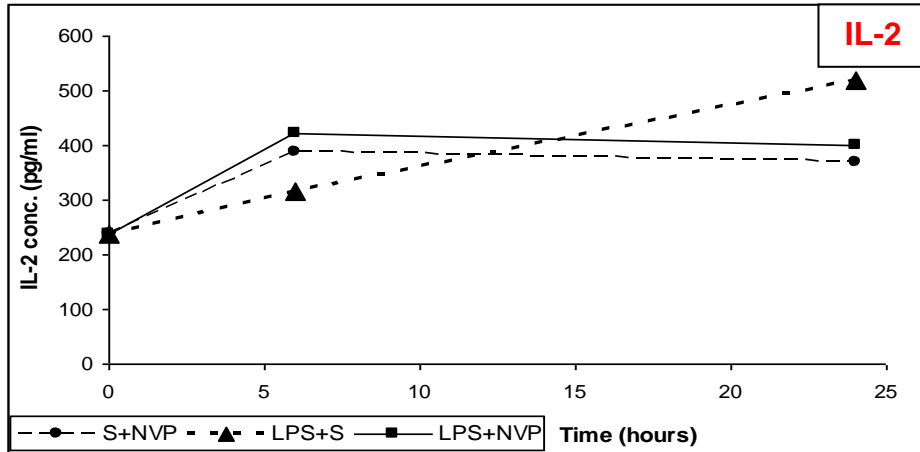


Figure 6.8 a): Mean serum IL-2 levels of groups D (S+NVP), E (LPS+S) and F (LPS+NVP) at 6 and 24 hours after dosing

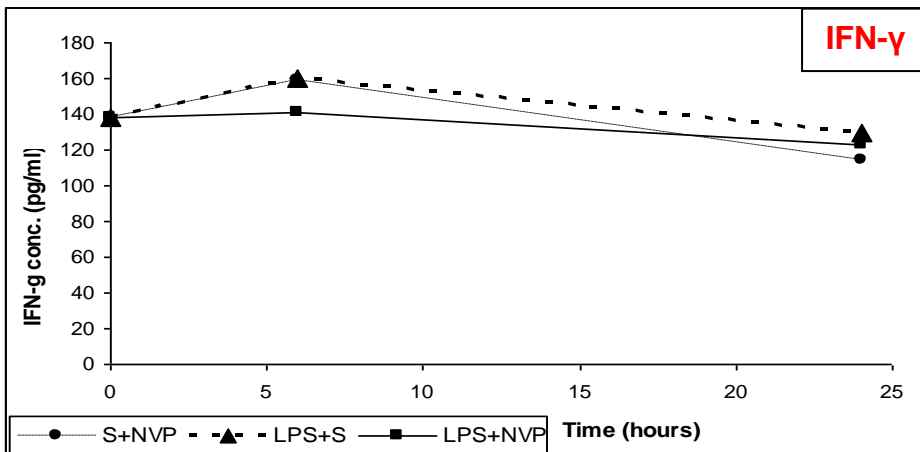


Figure 6.8 b): Mean serum IFN- γ levels of groups D (S+NVP), E (LPS+S) and F (LPS+NVP) at 6 and 24 hours after dosing

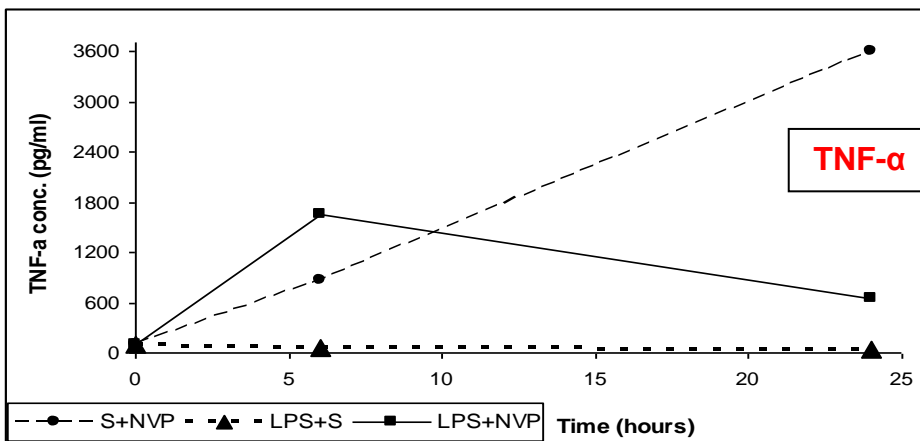


Figure 6.8 c): Mean serum TNF- α levels of groups D (S+NVP), E (LPS+S) and F (LPS+NVP) at 6 and 24 hours after dosing

6.7.2 d) Nevirapine plasma monitoring

Table 6.5 shows nevirapine plasma concentrations at 6 and 24 hours after dosing, while Figure 6.9 illustrates the trends of the nevirapine plasma concentrations. Figure 6.9 shows that, by 6 hours, nevirapine concentrations were lower in the LPS+NVP group than in the S+NVP group, probably due to slow absorption, but by 24 hours, this had been reversed in that nevirapine plasma concentrations were higher in the LPS+NVP group than in the S+NVP group (Table 6.5).

Table 6.5: Mean \pm SD values of nevirapine plasma concentrations of groups D (S+NVP) and F (LPS+NVP) at 6 and 24 hours after dosing

| Group (n = 5) | NVP concentration ($\mu\text{g/ml}$) |
|--------------------|--|
| D (S+NVP) | |
| 6 Hours | 3.65 ± 3.0 |
| 24 Hours | 0.49 ± 0.3 |
| F (LPS+NVP) | |
| 6 Hours | 1.31 ± 1.4 |
| 24 Hours | 2.40 ± 0.8 |

S = saline; NVP = nevirapine; LPS = lipopolysaccharide

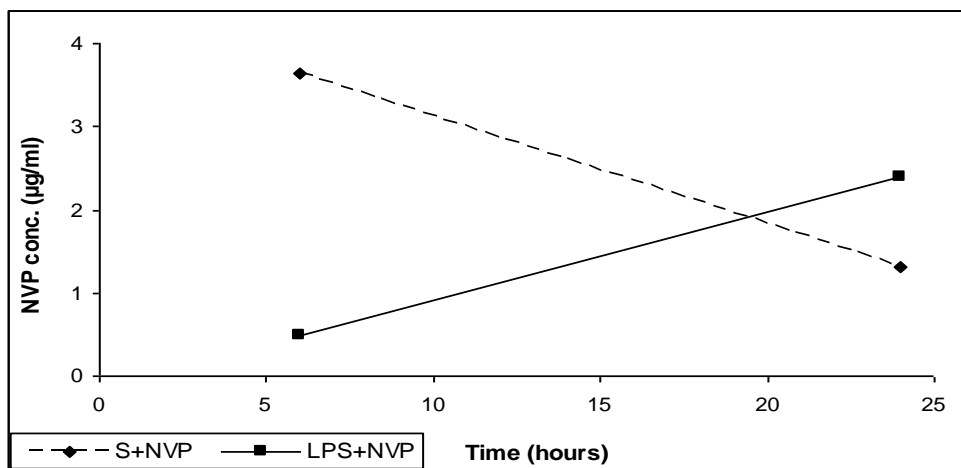


Figure 6.9: Graph of nevirapine plasma levels of groups D (S+NVP) and F (LPS+NVP) at 6 and 24 hours after dosing

6.7.2 e) Overall observation

- In the acute phase the saline+nevirapine (S+NVP) group induced hepatotoxicity that was characterised by intracellular histopathology changes, which were associated with increased TNF- α levels.
- The lipopolysaccharide+saline (LPS+S) group exhibited hepatotoxicity, characterised by extracellular histopathology changes and these were associated with increased IL-2 concentrations.
- In the group co-administered with LPS and nevirapine (LPS+NVP), there was less hepatotoxicity, despite increased levels of IL-2 and TNF- α .
- Nevirapine plasma concentrations at 24 hours were higher in the LPS+NVP group than in the S+NVP group.

6.7.3 Chronic phase

Table 6.6 shows the weights for animals during the chronic phase. There were more changes in weight in the LPS+NVP group than in the S+NVP group (Table 6.6). Because the changes were 5 – 10% of body weight, this was more likely due to mild-moderate dehydration in the past 24 hours because of loss of appetite caused by LPS induced immune sickness, as indicated under the direct observations (Section 6.7.1).

Table 6.6: Change in rat weights before and after treatment of untreated rats, and groups G (S+NVP) and H (LPS+NVP) over a 21 day dosing period

| Group (n = 5) | Weight (g) | | |
|--------------------------|-------------------|-----------------|---------------|
| | Before Rx | After Rx | Change |
| Untreated | | | |
| 7 Days | 392 ± 13 | 404 ± 12 | 12 ± 4 |
| 14 Days | 399 ± 14 | 419 ± 21 | 21 ± 10 |
| 21 Days | 376 ± 24 | 408 ± 30 | 37 ± 8 |
| G (S+NVP) | | | |
| 7 Days | 264 ± 5 | 274 ± 11 | 10 ± 7 |
| 14 Days | 260 ± 8 | 278 ± 32 | 17 ± 24 |
| 21 Days | 279 ± 14 | 310 ± 4 | 31 ± 11 |
| H (LPS+NVP) | | | |
| 7 Days | 271 ± 5 | 256 ± 13 | -11 ± 9 |
| 14 Days | 269 ± 7 | 277 ± 8 | 8 ± 4 |
| 21 Days | 272 ± 9 | 295 ± 13 | 23 ± 9 |

Rx = treatment; S = saline; NVP = nevirapine; LPS = lipopolysaccharide

6.7.3 a) *Hepatotoxicity*

Table 6.7 shows the liver function tests, while Figures 6.10 a – 6.10 g are the representative photographs of the liver pathology, and Table 6.8 is a tally of the main pathological lesions in the histopathology reports.

i) Liver function tests

In Table 6.7 the liver function test results of the two test groups did not show much difference from those of the control group ($P > 0.05$; Appendix I). Once again it was observed that the liver function tests were higher than quoted in the literature (Johnson-Delaney, 1996), however, fortunately this is normal in our setting as already discussed in Section 6.7.2 a i. Although increased ALT was observed on days 7 and 14 in the S+NVP group, this was not different from the control. In the LPS+NVP group, ALT levels were relatively high on day 7 in comparison to days 14 and 21, but these too were not different from the control, probably due to the big standard deviation value ($P > 0.05$; Appendix I). For all test groups (S+NVP and LPS+NVP), ALT levels were always lower by day 21. Of note, hepatotoxicity changes were observed in the S+NVP group on day 7 (Figure 6.10 b) versus a normal ALT (58.5 u/l). Therefore, as for the acute phase, there was no correlation between the ALT observations and the histopathology changes, as such, and from here on histopathology results were used as the indicator of hepatotoxicity.

ii) S+NVP group

From the histopathology results, the saline+nevirapine (S+NVP) group exhibited hepatotoxicity on day 7 (Figure 6.10 b), but normal histology on day 14 (Figure 6.10 c) and day 21 (Figure 6.10 d). From the report, nevirapine hepatotoxicity on day 7 was characterised by centrilobular hepatocellular degeneration and cell swelling with a cloudy appearance of the cytoplasm of hepatocytes, hepatocellular apoptosis, and prominent lymphoplasmacytic cuffing (Section 6.7.3 b ii, pg. 88). However, the liver injury dissipated with continual

administration of S+NVP, so that by days 14 and 21, there was no evidence of hepatotoxicity (Sections 6.7.3 b iii – 6.7.3 b iv, pg. 88 - 89). The lesions are also summarised in the tally table (Table 6.8).

iii) LPS+NVP group

From the histopathology results, the lipopolysaccharide+nevirapine (LPS+NVP) group exhibited normal histology at all occasions, *i.e.*, day 7, 14 and 21 (Figures 6.10 e – 6.10 g), illustrating that LPS attenuates the nevirapine induced hepatotoxicity (Sections 6.7.3 b v – 6.7.3 b vii, pg. 89 - 90). This is also as illustrated in the tally table (Table 6.8).

Table 6.7: Mean \pm SD values of liver function test results of untreated rats and groups G (S+NVP) and H (LPS+NVP) over a 21 day dosing period

| Group (n = 5) | Liver function tests (units/l) | | |
|--------------------|--------------------------------|-----------------|-----------------|
| | ALP | ALT | AST |
| Untreated | | | |
| 7 Days | 262.5 \pm 11 | 67.8 \pm 24 | 169.3 \pm 107 |
| 14 Days | 273.8 \pm 57 | 55.4 \pm 5 | 115.8 \pm 10 |
| 21 Days | 285.0 \pm 34 | 57.8 \pm 6 | 120.8 \pm 15 |
| G (S+NVP) | | | |
| 7 Days | 194.8 \pm 31 | 58.5 \pm 10 | 139.0 \pm 58 |
| 14 Days | 208.7 \pm 31 | 75.3 \pm 6 | 126.0 \pm 25 |
| 21 Days | 152.0 \pm 64 | 49.0 \pm 7 | 112.3 \pm 12 |
| H (LPS+NVP) | | | |
| 7 Days | 403.3 \pm 277 | 147.0 \pm 131 | 427.0 \pm 403 |
| 14 Days | 150.6 \pm 11 | 52.0 \pm 12 | 111.8 \pm 26 |
| 21 Days | 165.0 \pm 12 | 33.8 \pm 7 | 89.8 \pm 14 |

ALP = alkaline phosphatase; **ALT** = alanine aminotransferase; **AST** = aspartate aminotransferase; **S** = saline; **NVP** = nevirapine; **LPS** = lipopolysaccharide

6.7.3 b) *Histopathology photographs and reports*

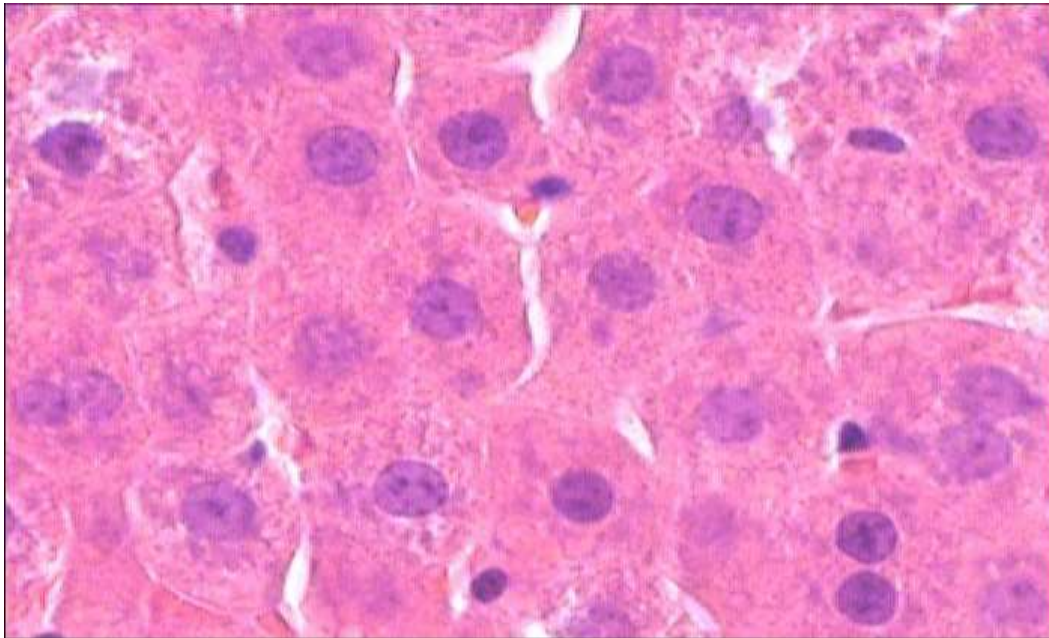


Figure 6.10 a): Liver section from an untreated rat at time 0 showing no lesions and necrosis, and normal portal tracts with no inflammation (Section 6.7.3 b i, pg. 88)

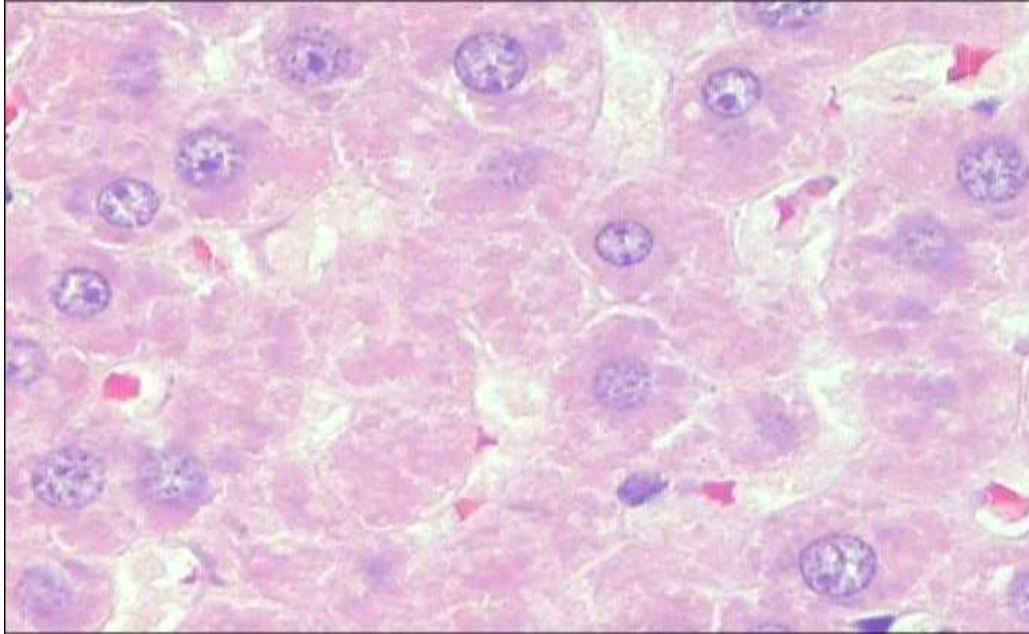


Figure 6.10 b): Liver section from the S+NVP group after 7 days of dosing, showing centrilobular hepatocellular degeneration, cell swelling, hepatocellular apoptosis, and prominent lymphoplasmacytic cuffing (Section 6.7.3 b ii, pg. 88)

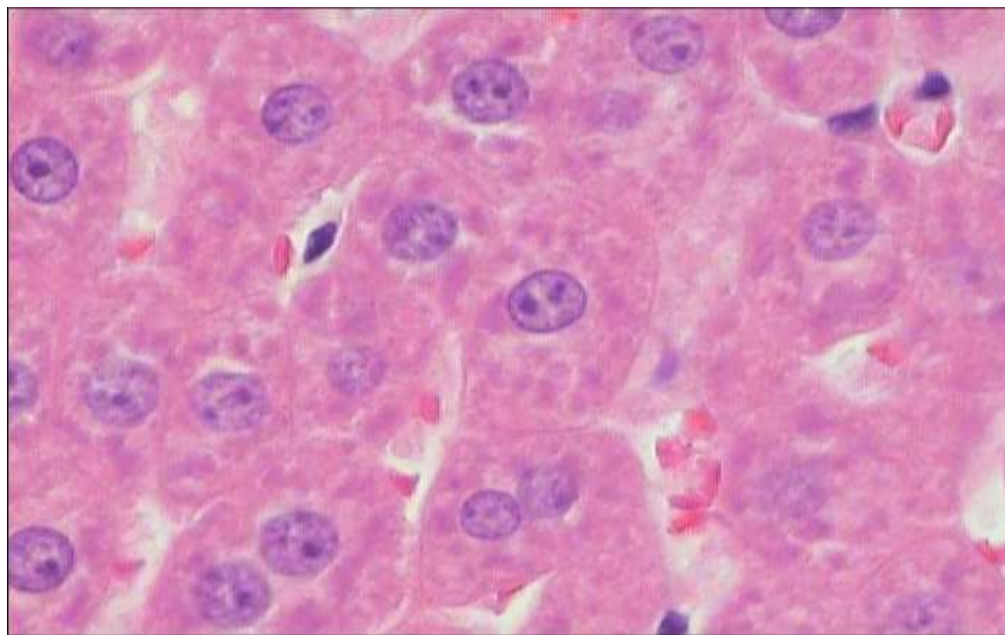


Figure 6.10 c): Liver section from the S+NVP group after 14 days of dosing, showing histologically normal parenchyma and sinusoids, and morphologically normal portal tracts (Section 6.7.3 b iii, pg. 88)

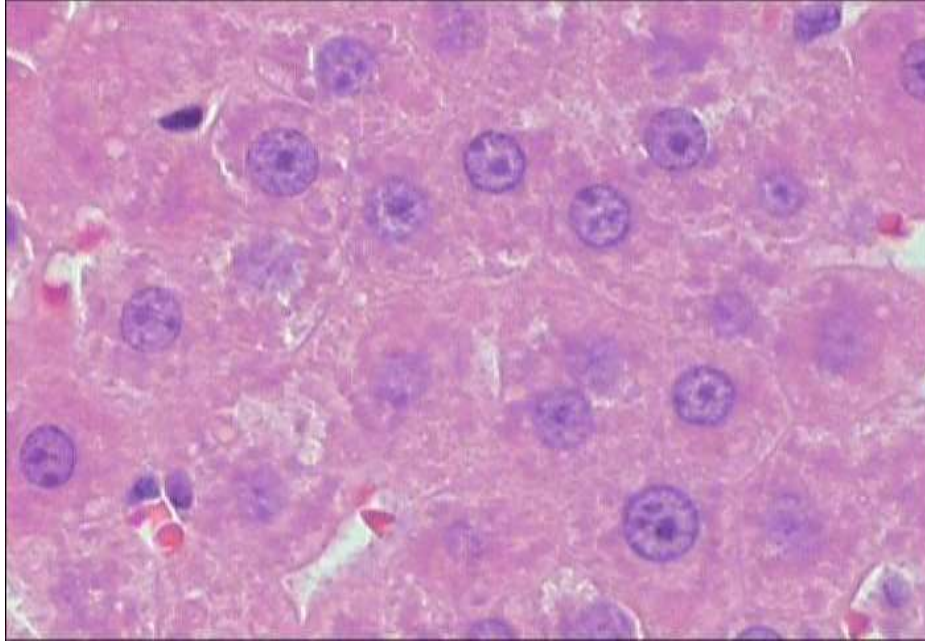


Figure 6.10 d): Liver section from the S+NVP group after 21 days of dosing, showing no lesions in the parenchyma and portal tracts (Section 6.7.3 b iv, pg. 89)

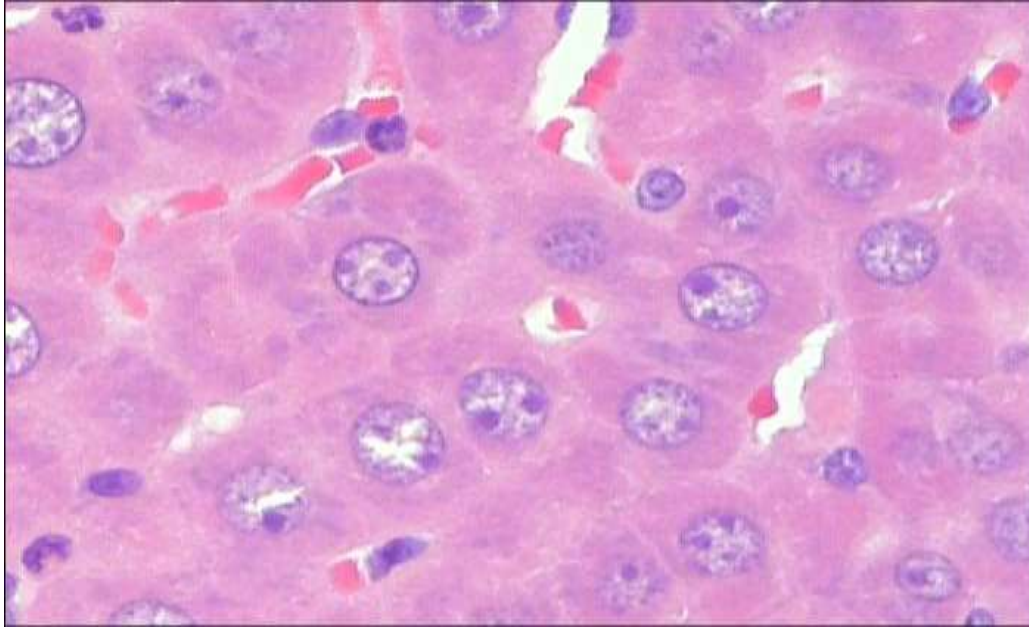


Figure 6.10 e): Liver section from the LPS+NVP group after 7 days of dosing, showing normal parenchymal and Kupffer cells, and moderate apoptosis (Section 6.7.3 b v, pg. 89)

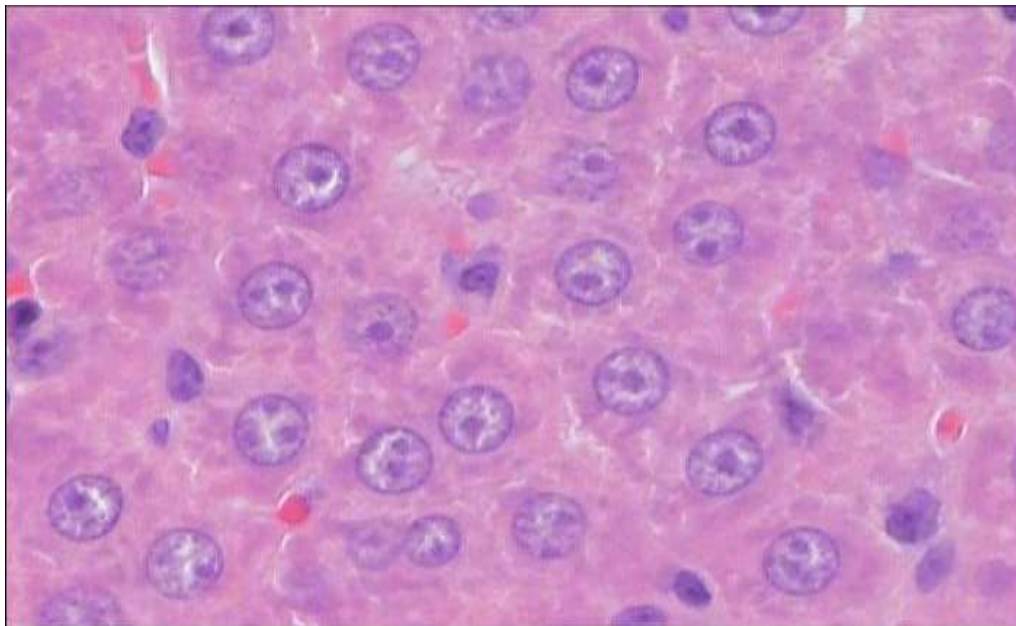


Figure 6.10 f): Liver section from the LPS+NVP group after 14 days of dosing, showing normal hepatocytes and the presence of Kupffer cells, as well as normal bile ducts (Section 6.7.3 b vi, pg. 89)

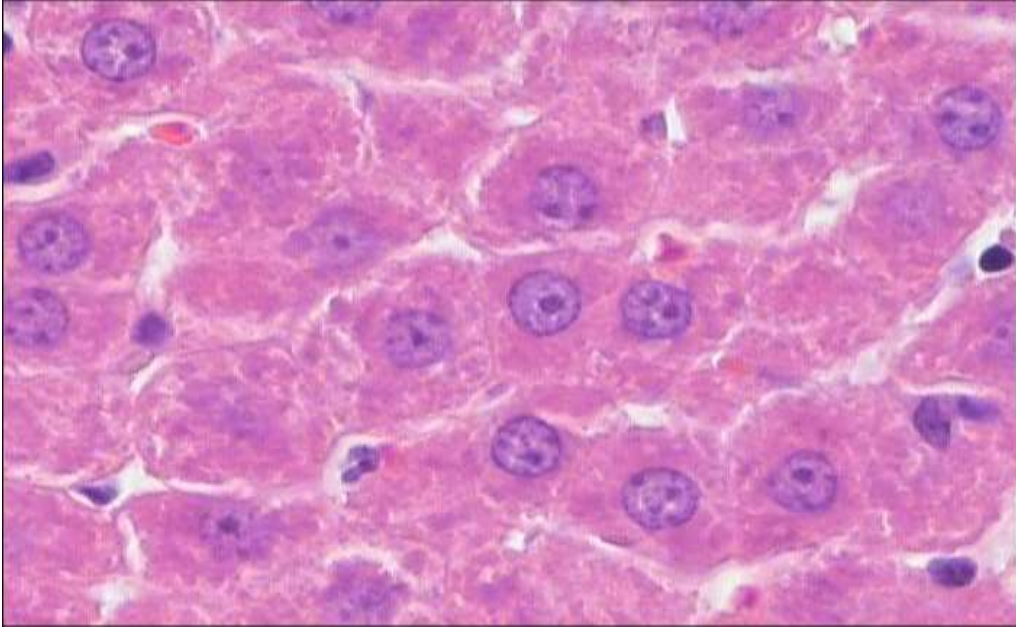


Figure 6.10 g): Liver section from the LPS+NVP group after 21 days of dosing, showing normal parenchymal hepatocytes and portal tracts (Section 6.7.3 b vii, pg. 90)

Figures 6.10 a – 6.10 g: Histopathology reports

Liver sections for histopathology (Figures 6.10 a – 6.10 g) were selected and the following diagnosis and remarks were made by the veterinary pathologist.

i) Figure 6.10 a: Liver section from an untreated rat at time 0

A representative photograph of a rat liver from an untreated rat. The corresponding pathology report: *“No lesions are observed in the liver parenchyma in the Haematoxylin and Eosin stained section. The portal tracts are normal without any inflammation visible. There is no hepatic parenchymal degeneration or necrosis detected.”*

ii) Figure 6.10 b: Liver section from the S+NVP group after 7 days of dosing

A representative photograph of a rat liver, 24 hours after dosing with saline and nevirapine in group G treated daily with nevirapine for 7 days. The corresponding pathology report: *“There appears to be mild centrilobular hepatocellular degeneration and cell swelling present with a cloudy appearance of the cytoplasm of hepatocytes in the central part of the liver lobules. On the periphery moderate hepatocellular apoptosis could be detected. The Kupffer cells are prominent. Within the portal tracts lymphocytic infiltrates are mild except for one blood vessel with prominent perivascular lymphoplasmacytic cuffing. No pathology is detected in the bile ducts.”*

iii) Figure 6.10 c: Liver section from the S+NVP group after 14 days of dosing

A representative photograph of a rat liver, 24 hours after dosing with saline and nevirapine in group G treated daily with nevirapine for 14 days. The corresponding pathology report: *“The parenchyma is histologically normal without any parenchymal cell histopathological changes present. The sinusoids are normal and normal apoptosis could be detected. There is no hepatocellular*

degeneration evident. The portal tracts appear morphologically normal in this liver section.”

iv) Figure 6.10 d: Liver section from the S+NVP group after 21 days of dosing

A representative photograph of a rat liver, 24 hours after dosing with saline and nevirapine in group G treated daily with nevirapine for 21 days. The corresponding pathology report: *“No lesions are visible within the parenchyma as well as the portal tracts in this specimen. There is no inflammation found. The portal areas are not fibrosed or chronically inflamed.”*

v) Figure 6.10 e: Liver section from the LPS+NVP group after 7 days of dosing

A representative photograph of a rat liver, 24 hours after dosing with LPS and nevirapine in group H treated daily with nevirapine for 7 days. The corresponding pathology report: *“Normal parenchymal cells are found and the macrophages (Kupffer cells) within the sinusoidal wall also appear normal. Apoptosis is moderate and scattered in the liver parenchyma. Mild lymphocytic infiltrates could be detected with perivascular distribution in the portal areas. There is no fibrosis detected.”*

vi) Figure 6.10 f: Liver section from the LPS+NVP group after 14 days of dosing

A representative photograph of a rat liver, 24 hours after dosing with LPS and nevirapine in group H treated daily with nevirapine for 14 days. The corresponding pathology report: *“The parenchymal cells (hepatocytes) appear normal while normal Kupffer cells could be detected within the hepatic sinusoids. In the portal tracts minimal lymphocytic infiltrates could be detected with perivascular distribution. The bile ducts appear normal.”*

vii) Figure 6.10 g: Liver section from the LPS+NVP group after 21 days of dosing
A representative photograph of a rat liver, 24 hours after dosing with LPS and nevirapine in group H treated daily with nevirapine for 21 days. The corresponding pathology report: *“The liver parenchymal hepatocytes appear morphologically normal without any degeneration or necrosis detected. There is no increased apoptosis visible. Normal-appearing portal tracts could be detected in the liver section.”*

Table 6.8: Tally of main pathology lesions in rat livers of groups G (S+NVP) and H (LPS+NVP) at 7, 14 and 21 days after dosing

| Pathology | S+NVP | | | LPS+NVP | | |
|----------------------------------|--------|---------|---------|---------|---------|---------|
| | 7 Days | 14 Days | 21 Days | 7 Days | 14 Days | 21 Days |
| Swollen cells | ++ | 0 | 0 | 0 | 0 | 0 |
| Portal tract inflammation | ++ | 0 | 0 | 0 | 0 | 0 |
| Apoptosis | ++ | 0 | 0 | ++ | 0 | 0 |
| Degenerative changes | + | 0 | 0 | 0 | 0 | 0 |
| Lymphoplasmacytic cuffing | + | 0 | 0 | + | + | 0 |

S = Saline; NVP = nevirapine; LPS = lipopolysaccharide

6.7.3 c) Immune response

i) Cytokines

Table 6.9 shows the changes in cytokine concentration profiles (mean \pm SD) on days 7, 14 and 21, while Figures 6.11 a – 6.11 c are the mean versus time plots of the cytokine profiles. Although not statistically significant, the concentrations of IL-2 and IFN- γ on the three occasions were higher in the saline+nevirapine (S+NVP) group compared to the lipopolysaccharide+nevirapine (LPS+NVP) group (Figures 6.11 a - 6.11 b; Table 6.9). Even though the TNF- α concentrations were higher in the LPS+NVP group than in the S+NVP group, the same trend was depicted by an increase on day 7, and a gradual decrease on days 14 and 21 (Figure 6.11 c; Table 6.9).

Table 6.9: Mean \pm SD values of serum cytokine levels of IL-2, IFN- γ and TNF- α of untreated rats, and groups G (S+NVP) and H (LPS+NVP) over 21 days of dosing

| Group (n = 5) | IL-2 | IFN- γ | TNF- α |
|--------------------|--------------------|-------------------|----------------------|
| Untreated | | | |
| Time 0 | 238.83 \pm 18.9 | 138.65 \pm 8.3 | 103.43 \pm 44.2 |
| G (S+NVP) | | | |
| 7 Days | 359.66 \pm 62.9 | 121.34 \pm 21.8 | 3547.11 \pm 5619.0 |
| 14 Days | 378.90 \pm 60.3 | 128.19 \pm 28.2 | 1239.07 \pm 628.2 |
| 21 Days | 469.35 \pm 143.1 | 167.33 \pm 58.4 | 769.09 \pm 137.5 |
| H (LPS+NVP) | | | |
| 7 Days | 298.76 \pm 10.8 | 103.13 \pm 34.6 | 5403.88 \pm 3709.3 |
| 14 Days | 351.97 \pm 41.1 | 109.24 \pm 27.4 | 2271.02 \pm 2251.0 |
| 21 Days | 313.08 \pm 23.2 | 124.27 \pm 15.4 | 1880.04 \pm 2401.2 |

IL-2 = interleukin-2; IFN- γ = interferon- γ ; TNF- α = tumour necrosis factor- α ; S = saline; NVP = nevirapine; LPS = lipopolysaccharide

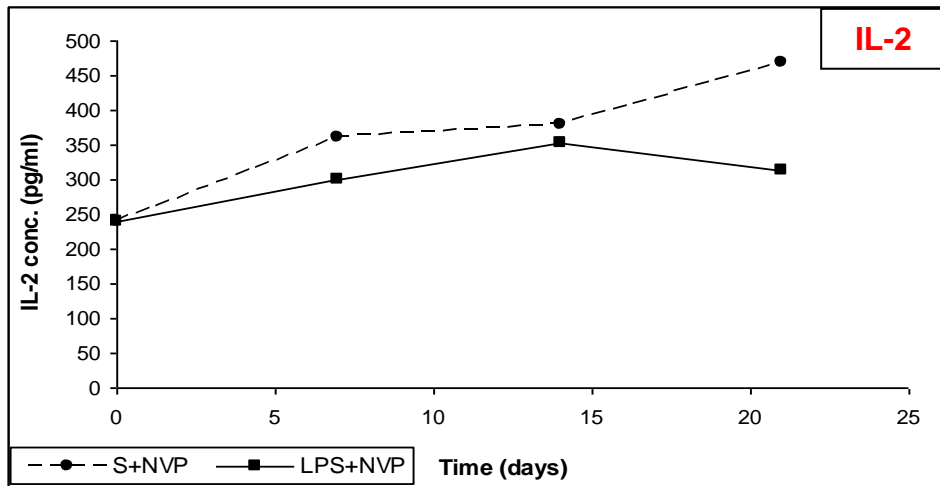


Figure 6.11 a): Mean serum IL-2 levels of groups G (S+NVP) and H (LPS+NVP) over a 21 day dosing period

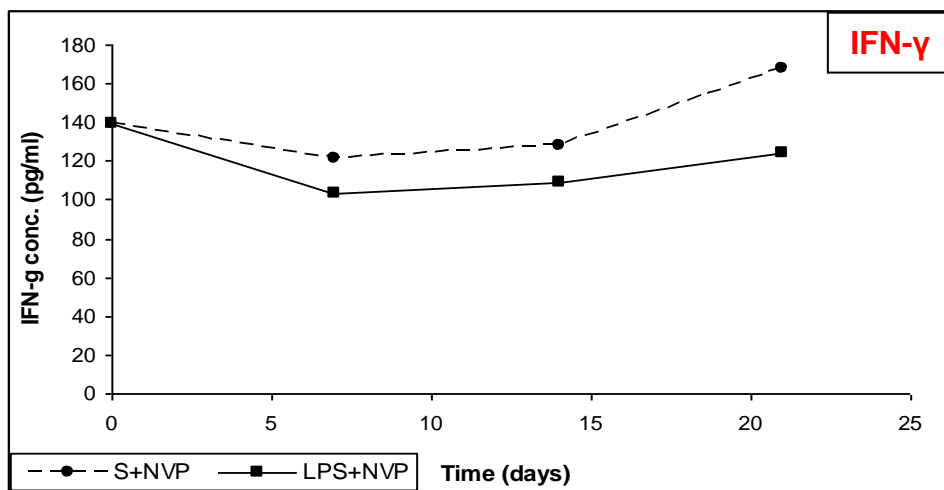


Figure 6.11 b): Mean serum IFN- γ levels of groups G (S+NVP) and H (LPS+NVP) over a 21 day dosing period

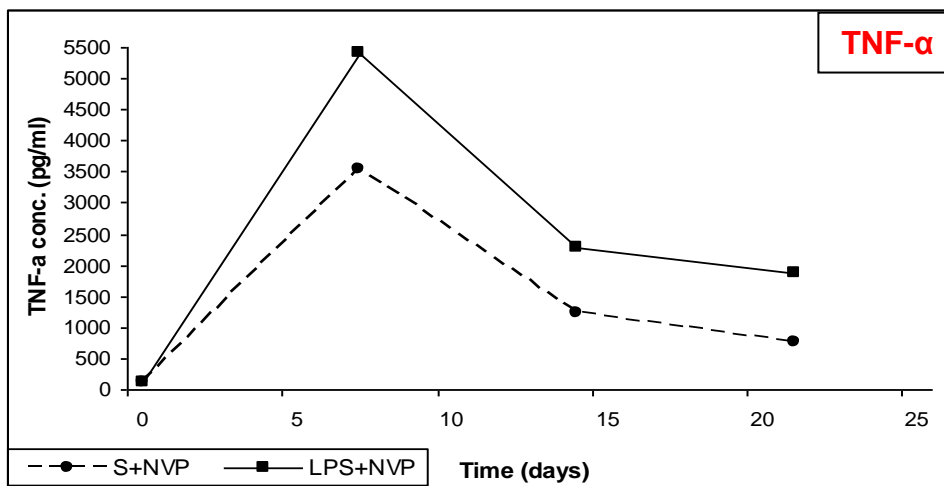


Figure 6.11 c): Mean serum TNF- α levels of groups G (S+NVP) and H (LPS+NVP) over a 21 day dosing period

ii) Haematology

Table 6.10 shows the full blood count values after 21 days of treatment. Whereas there was increased white cell count in both the saline+nevirapine (S+NVP) and lipopolysaccharide+nevirapine (LPS+NVP) groups, there was increased neutrophil count with moderate lymphocytosis in the LPS+NVP group, while the group administered with S+NVP exhibited profound lymphocytosis of almost 100%. There was no effect on other white cells. Surprisingly though, both groups exhibited thrombocytopenia (increased platelet count).

Table 6.10: Mean \pm SD values of full blood count results of groups G (S+NVP) and H (LPS+NVP) after 21 days of dosing

| Group | G (S+NVP) | H (LPS+NVP) | Reference value |
|-------------------------|--------------------------------------|-------------------------------------|-----------------------------------|
| Parameters | | | |
| Red cell count | 8.91 \pm 0.4 | 9.08 \pm 0.1 | 6.76 – 9.75 x 10 ¹² /l |
| Haemoglobin | 16.70 \pm 0.7 | 16.93 \pm 0.5 | 11.5 – 16.1 g/dL |
| Haematocrit | 0.74 \pm 0.0 | 0.73 \pm 0.0 | 39.6 – 52.5 % |
| MCV | 83.33 \pm 1.2 | 83.00 \pm 1.0 | 48 – 70 fl |
| MCH | 19 \pm 0.0 | 19.00 \pm 0.5 | 17.2 – 20.4 pg |
| White cell count | 10.90 \pm 0.5 | 15.33 \pm 2.1 | 6.6 – 12.6 x 10 ⁹ /l |
| Neutrophils | 2.22 \pm 0.6 | 8.89 \pm 1.3 | 1.77 – 3.38 x 10 ⁹ /l |
| Lymphocytes | 7.95 \pm 1.4 | 5.33 \pm 1.1 | 4.78 – 9.12 x 10 ⁹ /l |
| Monocytes | 0.59 \pm 0.1 | 0.74 \pm 0.5 | 0.01 – 0.04 x 10 ⁹ /l |
| Eosinophils | 0.10 \pm 0.0 | 0.57 \pm 0.5 | 0.03 – 0.08 x 10 ⁹ /l |
| Basophils | 0.10 \pm 0.0 | 0.00 \pm 0.0 | 0.00 – 0.003 x 10 ⁹ /l |
| Platelet count | 974.67 \pm 184.1 | 601.00 \pm 85.4 | 150 – 460 x 10 ⁹ /l |

S = saline; NVP = nevirapine; LPS = lipopolysaccharide; MCV = mean corpuscular volume; MCH = mean corpuscular haemoglobin (Johnson-Delaney, 1996; Giknis and Clifford, 2008)

6.7.3 d) Nevirapine plasma monitoring

Table 6.11 shows nevirapine plasma concentrations of the saline+nevirapine (S+NVP) and lipopolysaccharide+nevirapine (LPS+NVP) groups after 7, 14 and 21 days of dosing, while Figure 6.12 illustrates the trend of nevirapine plasma concentrations in the respective groups. Nevirapine plasma concentrations were consistently higher in the LPS+NVP group than in the S+NVP group, which indicates that, most probably, LPS inhibited nevirapine metabolism.

Table 6.11: Mean \pm SD values of nevirapine plasma levels of groups G (S+NVP) and H (LPS+NVP) after 7, 14 and 21 days of dosing

| Group | NVP concentration ($\mu\text{g/ml}$) |
|--------------------|--|
| G (S+NVP) | |
| 7 Days | 0.75 \pm 0.4 |
| 14 Days | 0.10 \pm 0.0 |
| 21 Days | 0.06 \pm 0.0 |
| H (LPS+NVP) | |
| 7 Days | 0.88 \pm 0.6 |
| 14 Days | 0.48 \pm 0.3 |
| 21 Days | 0.86 \pm 0.3 |

NVP = nevirapine; S = saline; LPS = lipopolysaccharide

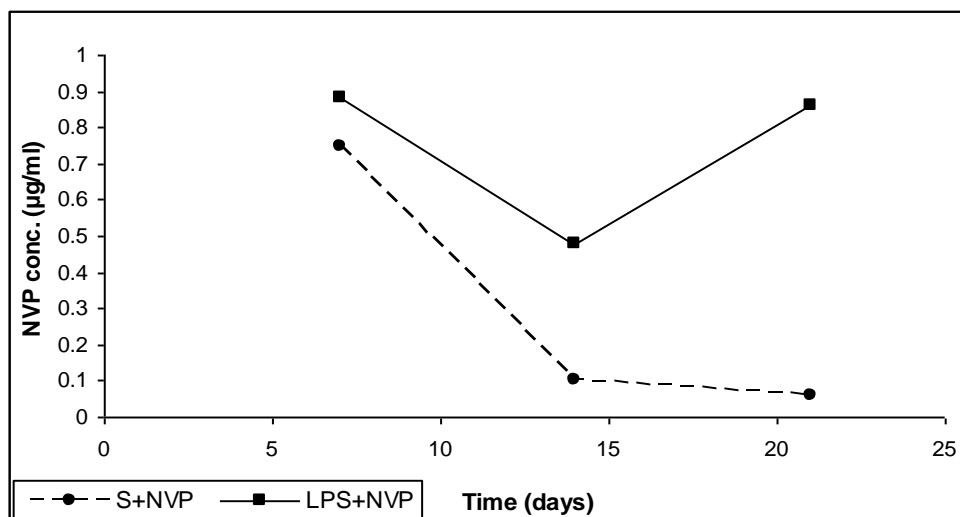


Figure 6.12: Graph of nevirapine plasma levels of groups G (S+NVP) and H (LPS+NVP) over 21 days

6.7.3 e) Overall observation

- In the chronic phase, the saline+nevirapine (S+NVP) group induced hepatotoxicity up to 7 days that was characterised by intra- and extracellular histopathology changes. However, the hepatotoxicity resolved by days 14 and 21 but this was associated with a progressive increase in IL-2 and IFN- γ levels, and lymphocytosis.
- The lipopolysaccharide+nevirapine (LPS+NVP) group did not exhibit hepatotoxicity on the three occasions, and this was associated with lower IL-2 and IFN- γ levels, and neutrophilia.
- Both groups exhibited thrombocytopenia.
- Nevirapine plasma concentrations were consistently higher in the LPS+NVP group than in the S+NVP group.

6.8 Discussion

6.8.1 Nevirapine hepatotoxicity

By observing for early changes in the liver and immune system during acute and chronic saline+nevirapine (S+NVP) administration to rats, it was shown that nevirapine induced hepatotoxicity is associated with immune stimulation. Nevirapine caused liver injury within hours of the first dose and this continued up to 7 days. However, by days 14 and 21 there was no evidence of hepatotoxicity, which implies that, with time, the body overcame the pathological process of nevirapine hepatotoxicity. Surprisingly, co-administration of lipopolysaccharide+nevirapine (LPS+NVP) prevented hepatotoxicity by either drug by an unknown mechanism. This interaction was not expected, because the study was designed to mimic a clinical situation in the first three weeks of single dose nevirapine therapy, albeit, HIV infection could not be induced. It was envisaged that since nevirapine induced hepatotoxicity is more common in patients with improved immune function (high CD4 count), this situation could be produced in a rat model by injection of a moderate dose of LPS to induce sub-clinical immune stimulation on selected days. It was therefore expected that LPS would augment nevirapine induced hepatotoxicity as it did for diclofenac and ranitidine induced

hepatotoxicity (Deng *et al.*, 2006 and Luyendyk *et al.*, 2003). Instead, LPS attenuated nevirapine induced hepatotoxicity. Nevertheless, this suggests that manipulation of the immune system has the potential to prevent nevirapine induced hepatotoxicity, and that understanding the mechanism involved may aid the development of an ultimate therapeutic agent.

6.8.2 Nevirapine immune stimulation

The increased neutrophil count associated with lipopolysaccharide+nevirapine administration signified increased phagocytosis and antigen presenting cells that can lead to release of cytokines and activation of the respective effector cells. On the other hand, saline+nevirapine administration selectively induced lymphocytosis, most probably due to a more refined single clone antigen. Unfortunately, the subgroup of lymphocytes affected was not investigated here. The thrombocytopenia observed in the LPS+NVP group correlates with the report by Pearson and co-workers (1995), which found that platelets accumulate in the liver after LPS administration, and that platelets were an important component of the mechanism of LPS induced liver injury. Interestingly, the same observation was made in the S+NVP group, implying that, most probably, nevirapine and LPS share the same platelet mediated mechanism of liver injury. Most important was that either pathways (or regimens) led to stimulation of the immune system as evidenced by increased T_{h1} cytokines (IL-2, IFN- γ and TNF- α). These cytokines activate macrophages and promote cell mediated immune responses against invasive intracellular pathogens, enhancing fever and tissue destruction (Dinarello, 2000). TNF- α promotes inflammation and apoptosis, while IL-2 increases the killing ability of NK cells and synthesis of other cytokines, including IFN- γ (Locksley *et al.*, 2001). Furthermore, together with TNF- α , IL-2 controls the induction of both T_{h1} and T_{h2} -responses by promoting T cell division and antibody synthesis by B cells (Kulmatycki and Jamali, 2005). IFN- γ is produced by NK and T cells. It has antiviral activities as it activates the pathway that leads to induction of cytotoxic T cells and augments TNF activity. It induces nitric oxide (NO) by NO synthetase which mediates apoptosis of damaged cells and killing of bacteria by macrophages (Opal and

De Palo, 2000; Dinarello, 2000). In effect, the three cytokines (IL-2, IFN- γ and TNF- α) mediate the destructive process of the body's defence mechanism, and both nevirapine and LPS, independent of each other, led to stimulation of this defence mechanism. Whereas for LPS, it was necessary defence against bacterial infection, it is not clear why (and how) nevirapine stimulated this defence mechanism.

In addition, it appears that the release of these T_h1 cytokines was more immediate with LPS than with nevirapine. Specifically, co-administration of lipopolysaccharide+nevirapine in the acute phase led to higher IL-2 concentrations than co-administration of saline+nevirapine, but this was reversed during chronic administration where IL-2 concentrations continued to rise in the S+NVP group while they remained static in the LPS+NVP group. This implies that nevirapine is a slow onset immune stimulant, whose effects increased only with chronic administration.

6.8.3 Possible mechanism of toxicity

Since continual stimulation of the immune system (IL-2, IFN- γ and TNF- α) by saline+nevirapine was not associated with progression of nevirapine induced hepatotoxicity, particularly after 7 days, it seems to dispel claims that the immune system has a role in nevirapine induced hepatotoxicity. Unfortunately, owing to a recent report on the metabolic activation of nevirapine to metabolites (Srivastava *et al.*, 2010), the changes in nevirapine concentrations suggested otherwise. They suggest that nevirapine was metabolised to immunogenic metabolites (or metabolic adducts) that triggered a cell mediated immune response, which then led to destruction or programmed elimination of hepatocytes (apoptosis) expressing the metabolic adducts (Holt and Ju, 2006). Accordingly, the lower concentrations of nevirapine in the S+NVP group implied that more nevirapine was metabolised to the immunogenic metabolites leading to increased immune stimulation and hepatotoxicity, while the higher nevirapine concentrations in the LPS+NVP group implied that less nevirapine was metabolised to the immunogenic metabolites, and therefore little or no hepatotoxicity occurred. In effect, LPS

attenuated nevirapine induced hepatotoxicity by inhibiting the metabolism of nevirapine to immunogenic metabolites, as well as by increased phagocytosis of the immunogenic metabolites.

This observation conforms to a previous report where nevirapine hepatotoxicity was associated with CYP3A induction (Walubo *et al.*, 2006). It means that increased metabolism of nevirapine due to auto-enzyme induction within the first 2 weeks could be responsible for perpetuating this immune toxicity and/or hepatotoxicity. The subsequent recovery from hepatotoxicity is because, within 1 – 2 weeks of nevirapine therapy, enzyme induction usually reaches maximum and the body processes are adjusted to eliminate the metabolic adducts faster, leading to amelioration of the hepatotoxicity. As such, nevirapine metabolic adducts are responsible for maintaining the immune stimulation, and this could explain the persistent increase in IL-2 and IFN- γ levels in the S+NVP group on chronic nevirapine administration, but this remains to be proven.

The mechanism by which the LPS led to higher concentrations of nevirapine is yet to be discerned. It could have been due to slowed absorption of nevirapine, but this would also have led to lower bioavailability, which would lead to lower concentrations of nevirapine. As such, the most plausible explanation is that LPS inhibited nevirapine metabolism. Indeed, several reports have shown that administration of LPS was associated with down regulation of cytochrome P450 activity in rats and that many cytokines are inhibitors of cytochrome P450 activity (Stanley, *et al.*, 1988; Morgan, 1989; Joeng, 2001). Likewise, the mechanism by which nevirapine ameliorated the LPS induced hepatotoxicity was not clear, but it could be due to competitive interference by nevirapine immunogenic metabolites for the immune system.

6.8.4 Comparative view

The findings of the current study have a lot in common with those reported for nevirapine induced skin reactions. In the current study, nevirapine 200 mg/kg was used after lower doses led to undetectable nevirapine concentrations by

24 hours. This concurred with earlier reports in which, at the same dose of nevirapine 150 mg/kg, there were fewer skin lesions (20%) and lower nevirapine concentrations in SD rats compared to the 100% in Brown Norwegian (BN) rats (Shenton *et al.*, 2003). They suggested that this was because SD rats metabolised nevirapine faster than the BN rats. Of note, in this study, male SD rats were used to minimise the occurrence of skin reactions observed in female rats from interfering with progression of the study. In another report, nevirapine induced skin reactions were prevented by inhibition of cytochrome P450 with aminobenzotriazole as well as the use of low dose nevirapine. It was suggested that, most probably, this was due to reduced formation of the 12-hydroxy-nevirapine metabolites (Chen *et al.*, 2009; Uetrecht, 2006). Furthermore, nevirapine induced skin reactions were prevented by using the immunosuppressant drug cyclosporine (Shenton *et al.*, 2005). Since cyclosporine is a known inhibitor of IL-2 production, information from the current study would help to explain cyclosporine effects as due to inhibition of IL-2 production, and consequently the cell mediated immunity response, thereby inhibiting activation of the apoptotic process. Therefore, cyclosporine antagonised nevirapine's stimulation of IL-2 production. On the other hand, the association of increased CD4 lymphocytes with the occurrence of skin reactions by Shenton *et al.* (2005) corroborates the selective lymphocytosis observed in the current study. In effect, the nevirapine induced hepatotoxicity observed in the current study is most probably similar to that observed in humans.

Lastly, this study also highlights the fact that observations of the immune system after single dose administration of drugs should never be used to draw conclusions about the effects of drugs during chronic drug therapy. Specifically in this case, stimulation of IL-2 production by nevirapine could not have been confirmed without chronic dosing.

6.8.5 Conclusion

In conclusion, it has been demonstrated that nevirapine is a slow onset immune stimulant, that nevirapine induced hepatotoxicity is associated with immune stimulation by nevirapine itself, and that LPS prevents nevirapine induced hepatotoxicity. These observations suggest that manipulation of the immune system may be one way to prevent nevirapine induced hepatotoxicity.

CONCLUSION AND FUTURE STUDIES

The objectives of this study were achieved as follows:

1. A method for the determination of nevirapine in a small plasma volume by high performance liquid chromatography was successfully developed. It was used to measure nevirapine plasma concentrations in rats treated with 200 mg/kg nevirapine for 6 and 24 hours (acute phase), and 7, 14 and 21 days (chronic phase).
2. The immune system contributed to nevirapine induced hepatotoxicity as observed by increased IL-2 and TNF- α in the acute phase, and increased IL-2, IFN- γ and TNF- α in the chronic phase.
 - Nevirapine is a slow onset immune stimulant.
 - Nevirapine induced hepatotoxicity is associated with immune stimulation by nevirapine itself.
 - LPS prevents nevirapine induced hepatotoxicity.

Implications:

3. Manipulation of the immune system may lead to the prevention of nevirapine induced hepatotoxicity.

Future studies:

4. Regarding the immune system, it is necessary to determine how nevirapine stimulates the cell mediated immune response in order to develop a starting point for preventative strategies.
5. There is a need to study the interaction between nevirapine and LPS when administered concomitantly to establish the mechanism by which LPS prevents nevirapine induced hepatotoxicity. This will aid in the search for drugs with similar effects on nevirapine, other than LPS, which are not of bacterial origin which may lead to additional infection.
6. Lastly, there is a need to establish methods of immune manipulation by drugs, other than LPS, to prevent nevirapine induced hepatotoxicity.

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APPENDICES

APPENDIX A: HPLC CALIBRATIONS OF NEVIRAPINE OVER 5 DAYS

Appendix A-1: Calibration, day 1

Table A-1: Calibration data, day 1

| Concentration ($\mu\text{g/ml}$) | Ratio |
|------------------------------------|-------|
| 1 | 0.033 |
| 3 | 0.055 |
| 5 | 0.083 |
| 8 | 0.114 |
| 10 | 0.132 |

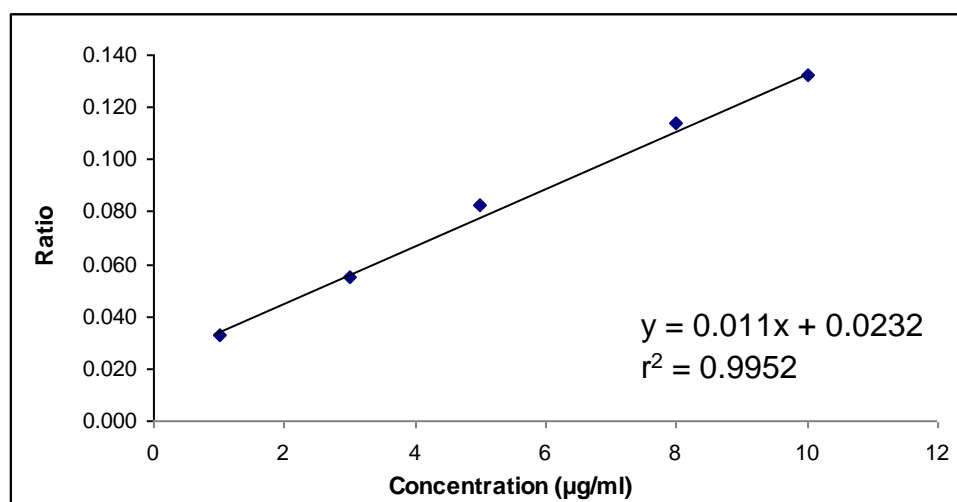


Figure A-1: Calibration curve, day 1

Appendix A-2: Calibration, day 2

Table A-2: Calibration data, day 2

| Concentration ($\mu\text{g/ml}$) | Ratio |
|------------------------------------|-------|
| 1 | 0.029 |
| 3 | 0.057 |
| 5 | 0.077 |
| 8 | 0.119 |
| 10 | 0.146 |

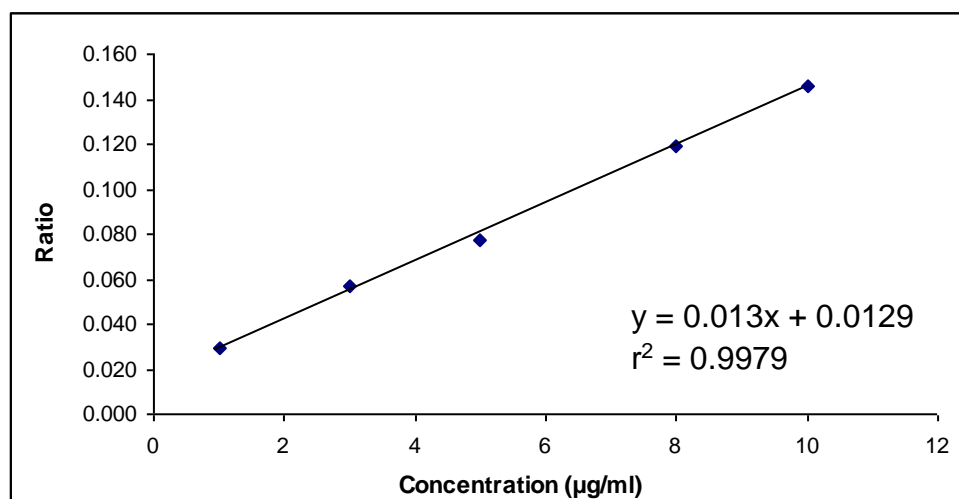


Figure A-2: Calibration curve, day 2

Appendix A-3: Calibration, day 3

Table A-3: Calibration data, day 3

| Concentration (µg/ml) | Ratio |
|-----------------------|-------|
| 1 | 0.033 |
| 3 | 0.058 |
| 5 | 0.080 |
| 8 | 0.114 |
| 10 | 0.137 |

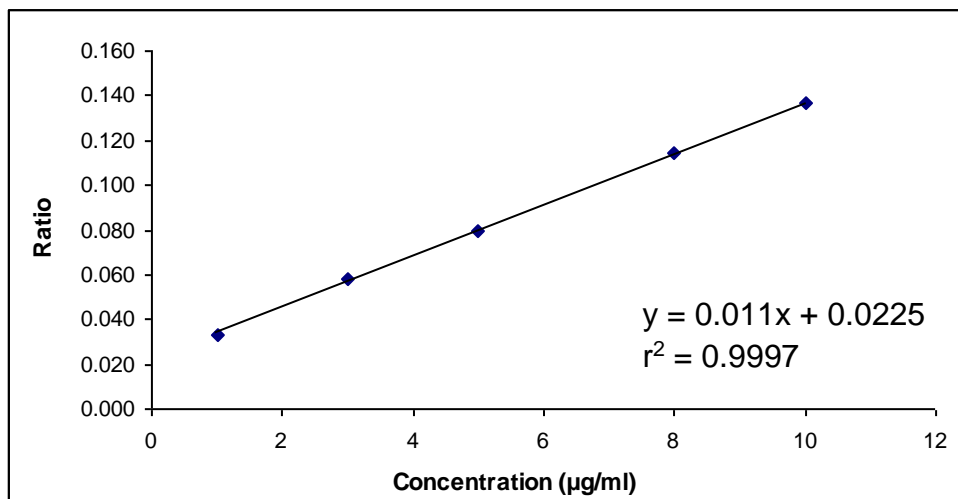


Figure A-3: Calibration curve, day 3

Appendix A-4: Calibration, day 4

Table A-4: Calibration data, day 4

| Concentration ($\mu\text{g/ml}$) | Ratio |
|------------------------------------|-------|
| 1 | 0.030 |
| 3 | 0.053 |
| 5 | 0.079 |
| 8 | 0.110 |
| 10 | 0.146 |

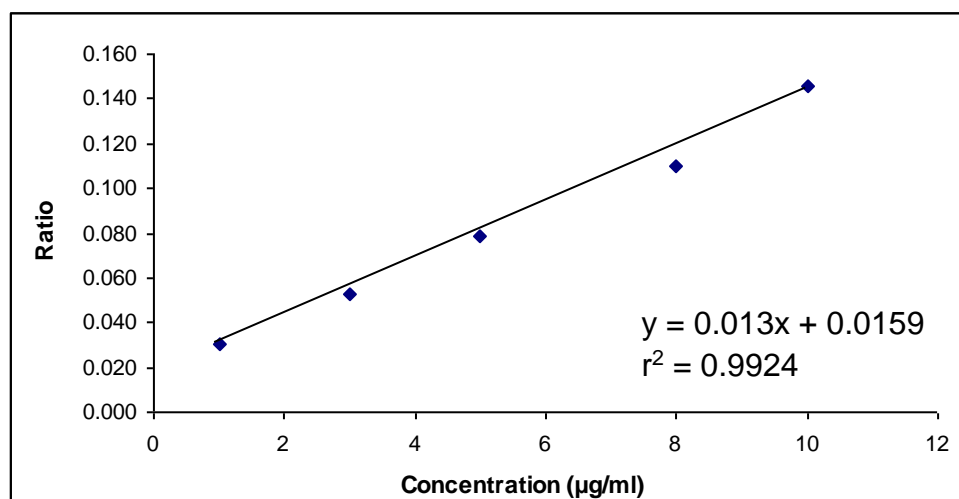


Figure A-4: Calibration curve, day 4

Appendix A-5: Calibration, day 5

Table A-5: Calibration data, day 5

| Concentration ($\mu\text{g/ml}$) | Ratio |
|------------------------------------|-------|
| 1 | 0.027 |
| 3 | 0.052 |
| 5 | 0.072 |
| 8 | 0.109 |
| 10 | 0.139 |

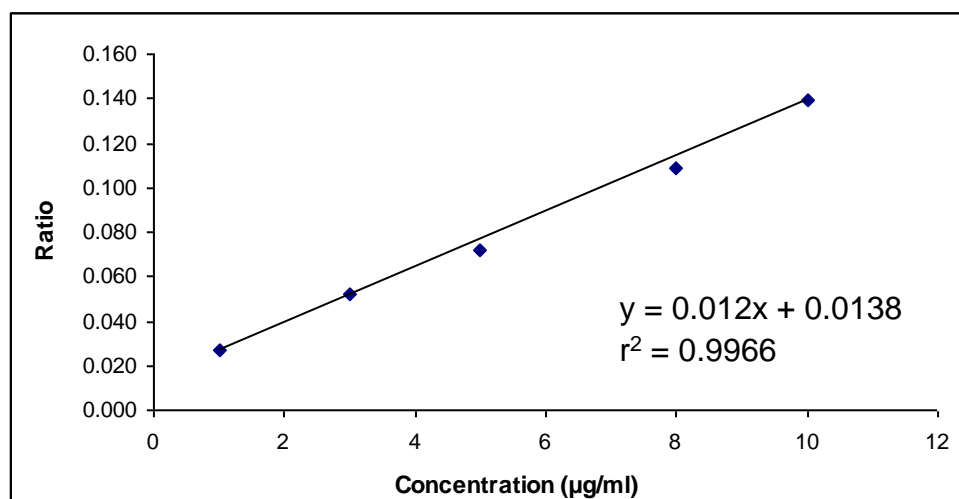


Figure A-5: Calibration curve, day 5

APPENDIX B: ACCURACY DETERMINATION OF NEVIRAPINE

Appendix B-1: Accuracy of nevirapine HPLC assay

Table B-1: Accuracy data of nevirapine HPLC assay

| Prep Conc. (µg/ml) | Meas Conc. 1 (µg/ml) | Meas Conc. 2 (µg/ml) | Meas Conc. 3 (µg/ml) | Meas Conc. 4 (µg/ml) | Meas Conc. 5 (µg/ml) | Mean | SD | % Acc | CV% |
|--------------------------|-------------------------------|-------------------------------|-------------------------------|-------------------------------|-------------------------------|-------------|-----|----------|------|
| 1 | 0.82 | 1.15 | 0.77 | 0.79 | 0.96 | 0.89 | 0.2 | 89.9 | 17.7 |
| 5 | 4.93 | 4.84 | 4.25 | 6.20 | 4.93 | 5.03 | 0.7 | 100.6 | 14.2 |
| 10 | 10.29 | 9.61 | 9.90 | 9.61 | 8.73 | 9.63 | 0.6 | 96.3 | 6.0 |

SD = standard deviation; **Acc** = accuracy; **CV%** = coefficient of variation

APPENDIX C: STABILITY DETERMINATION OF NEVIRAPINE

Appendix C-1: Stability of nevirapine at room temperature

Table C-1: Table of stability data at room temperature at 8, 12 and 24 hours

| Time | Prep. Conc. ($\mu\text{g/ml}$) | Measured Conc. 1 ($\mu\text{g/ml}$) | Measured Conc. 2 ($\mu\text{g/ml}$) | Mean | SD | %Stability | CV% |
|----------|----------------------------------|---------------------------------------|---------------------------------------|-------------|-----|------------|------|
| 8 Hours | 5 | 6.71 | 7.07 | 6.89 | 0.3 | 137.9 | 3.7 |
| 12 Hours | 5 | 6.76 | 6.64 | 6.70 | 0.1 | 134.0 | 1.3 |
| 24 Hours | 5 | 6.24 | 5.25 | 5.74 | 0.7 | 114.8 | 12.4 |

SD = standard deviation; CV% = coefficient of variation

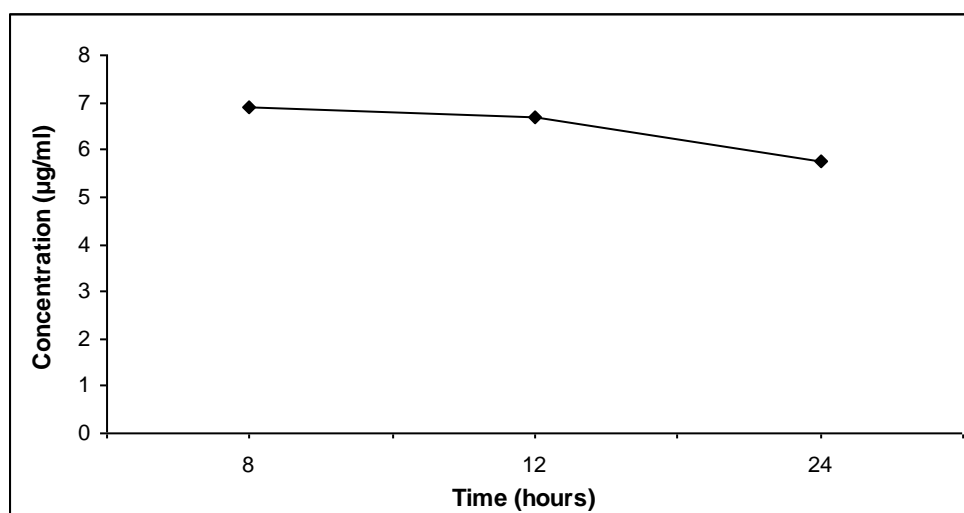


Figure C-1: Plot of stability of nevirapine at room temperature over time

Appendix C-2: Stability of nevirapine at 4°C

Table C-1: Table of stability data at 4°C at 8, 12 and 24 hours

| Time | Prep. Conc. (µg/ml) | Measured Conc. 1 (µg/ml) | Measured Conc. 2 (µg/ml) | Mean | SD | %Stability | CV% |
|----------|---------------------|--------------------------|--------------------------|-------------|-----|------------|-----|
| 8 Hours | 5 | 5.50 | 6.08 | 5.79 | 0.4 | 115.8 | 7.0 |
| 12 Hours | 5 | 5.62 | 5.93 | 5.77 | 0.2 | 115.4 | 3.8 |
| 24 Hours | 5 | 5.41 | 5.27 | 5.34 | 0.1 | 106.7 | 1.9 |

SD = standard deviation; CV% = coefficient of variation

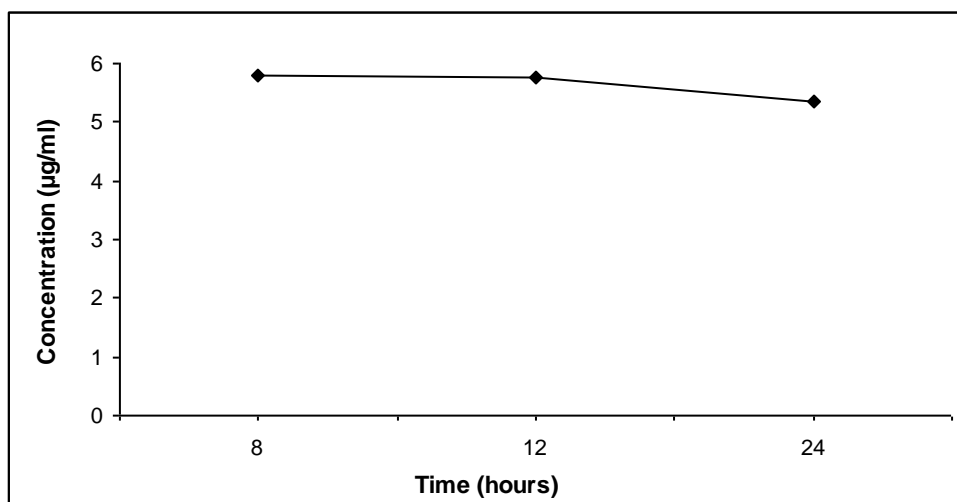


Figure C-2: Plot of stability of nevirapine at 4°C over time

Appendix C-3: Stability of nevirapine at -20°C

Table C-1: Table of stability data at -20°C at 8, 12 and 24 hours, and 1 week

| Time | Prep. conc. (µg/ml) | Measured conc. 1 (µg/ml) | Measured conc. 2 (µg/ml) | Mean | SD | %Stability | CV % |
|----------|---------------------|--------------------------|--------------------------|-------------|-----|------------|------|
| 8 Hours | 5 | 6.02 | 6.62 | 6.32 | 0.4 | 126.4 | 6.8 |
| 12 Hours | 5 | 5.34 | 7.78 | 6.56 | 1.7 | 131.2 | 26.4 |
| 24 Hours | 5 | 5.24 | 3.30 | 4.58 | 0.9 | 91.7 | 20.2 |
| 1 Week | 5 | 5.99 | 2.62 | 4.30 | 2.4 | 86.1 | 55.5 |

SD = standard deviation; CV% = coefficient of variation

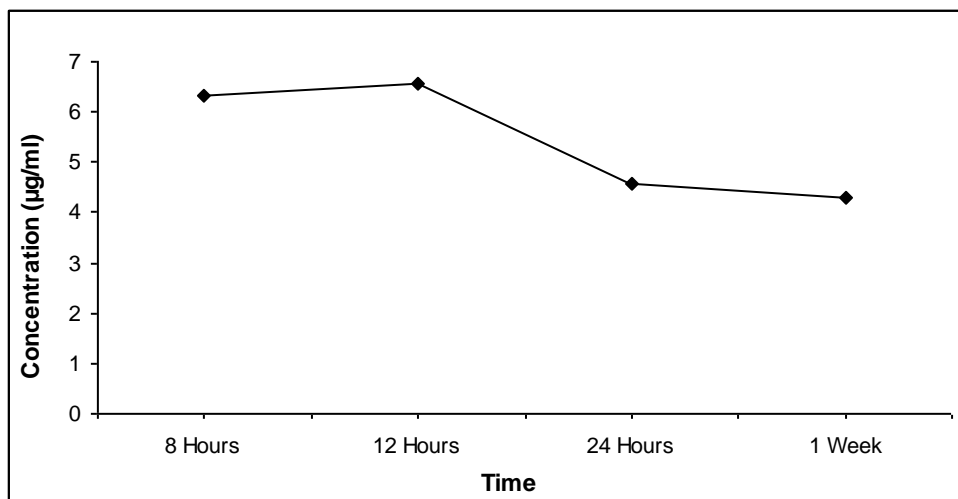


Figure C-3: Plot of stability of nevirapine at -20°C over time

APPENDIX D: RAT WEIGHTS

Appendix D-1: Rat weights during the preliminary experiment

Table D-1: Rat weights of groups A (saline), B (NVP low) and C (NVP high) over 28 days

| Group | Time | Rat 1 | Rat 2 | Rat 3 | Rat 4 | Rat 5 | Mean | SD |
|---------------------|-----------|----------|----------|----------|----------|----------|------------|----|
| A (saline) | | | | | | | | |
| 0 Days | Before Rx | 397 | 389 | 388 | 376 | 411 | 392 | 13 |
| 7 Days | After Rx | 413 | 404 | 401 | 386 | 416 | 404 | 12 |
| 0 Days | Before Rx | 386 | 382 | 400 | 415 | 410 | 399 | 14 |
| 14 Days | After Rx | 415 | 385 | 424 | 442 | 430 | 419 | 21 |
| 0 Days | Before Rx | 367 | 378 | 386 | 373 | 375 | 376 | 7 |
| 21 Days | After Rx | 395 | 394 | 415 | 385 | 398 | 397 | 11 |
| 0 Days | Before Rx | 392 | 388 | 350 | 340 | 383 | 371 | 24 |
| 28 Days | After Rx | 440 | 430 | 376 | 375 | 417 | 408 | 30 |
| B (NVP low) | | | | | | | | |
| 0 Days | Before Rx | 362 | 403 | 342 | 364 | 402 | 375 | 27 |
| 7 Days | After Rx | 363 | 422 | 354 | 396 | 412 | 389 | 30 |
| 0 Days | Before Rx | 378 | 389 | 355 | 363 | 368 | 371 | 13 |
| 14 Days | After Rx | 387 | 410 | 385 | 371 | 378 | 386 | 15 |
| 0 Days | Before Rx | 336 | 362 | 376 | 351 | 357 | 356 | 15 |
| 21 Days | After Rx | 360 | 371 | 399 | 364 | 388 | 376 | 17 |
| 0 Days | Before Rx | 366 | 357 | 356 | 351 | 362 | 358 | 6 |
| 28 Days | After Rx | 396 | 379 | 392 | 371 | 394 | 386 | 11 |
| C (NVP high) | | | | | | | | |
| 0 Days | Before Rx | 376 | 377 | 344 | 356 | 336 | 358 | 18 |
| 7 Days | After Rx | 370 | 402 | 353 | 366 | 349 | 368 | 21 |
| 0 Days | Before Rx | 397 | 379 | 359 | 319 | 335 | 358 | 31 |
| 14 Days | After Rx | 420 | 387 | 373 | 329 | 351 | 372 | 32 |
| 0 Days | Before Rx | 350 | 352 | 343 | 337 | 364 | 349 | 10 |
| 21 Days | After Rx | 378 | 381 | 370 | 357 | 385 | 372 | 11 |
| 0 Days | Before Rx | 358 | 390 | 340 | 357 | 376 | 362 | 19 |
| 28 Days | After Rx | 386 | 430 | 372 | 389 | 397 | 395 | 22 |

SD = standard deviation; NVP = nevirapine; Rx = treatment

Appendix D-2: Rat weights during the acute phase

Table D-2: Rat weights of groups D (S+NVP), E (LPS+S) and F (LPS+NVP) at the time of dosing

| Group | Rat 1 | Rat 2 | Rat 3 | Rat 4 | Rat 5 | Mean | SD |
|--------------------|-------|-------|-------|-------|-------|------------|----|
| D (S+NVP) | | | | | | | |
| 6 Hours | 392 | 380 | 327 | 385 | 374 | 372 | 26 |
| 24 Hours | 380 | 388 | 391 | 312 | 364 | 367 | 32 |
| E (LPS+S) | | | | | | | |
| 6 Hours | 281 | 336 | 290 | 291 | 310 | 302 | 22 |
| 24 Hours | 270 | 301 | 258 | 299 | 301 | 286 | 20 |
| F (LPS+NVP) | | | | | | | |
| 6 Hours | 384 | 377 | 391 | 349 | 392 | 379 | 18 |
| 24 Hours | 385 | 392 | 372 | 385 | 393 | 385 | 8 |

SD = standard deviation; **S** = saline; **NVP** = nevirapine; **LPS** = lipopolysaccharide

Appendix D-3: Rat weights during the chronic phase

Table D-3: Rat weights of groups G (S+NVP) and H (LPS+NVP) over 21 days

| Group | Time | Rat 1 | Rat 2 | Rat 3 | Rat 4 | Rat 5 | Mean | SD |
|--------------------|-----------|-------|-------|-------|-------|-------|------------|----|
| G (S+NVP) | | | | | | | | |
| 0 Days | Before Rx | 267 | 258 | 269 | 263 | - | 264 | 5 |
| 7 Days | After Rx | 274 | 263 | 289 | 269 | - | 274 | 11 |
| 0 Days | Before Rx | 269 | 258 | 254 | - | - | 260 | 8 |
| 14 Days | After Rx | 311 | 274 | 248 | - | - | 278 | 32 |
| 0 Days | Before Rx | 282 | 291 | 263 | - | - | 279 | 14 |
| 21 Days | After Rx | 309 | 314 | 307 | - | - | 310 | 4 |
| H (LPS+NVP) | | | | | | | | |
| 0 Days | Before Rx | 267 | 265 | 271 | 274 | 278 | 271 | 5 |
| 7 Days | After Rx | 245 | 267 | 268 | 273 | -* | 263 | 12 |
| 0 Days | Before Rx | 273 | 279 | 263 | 262 | 267 | 269 | 7 |
| 14 Days | After Rx | 285 | 287 | 273 | 267 | 274 | 277 | 8 |
| 0 Days | Before Rx | 284 | 269 | 263 | 266 | 279 | 272 | 9 |
| 21 Days | After Rx | 303 | 292 | 376 | 292 | 311 | 295 | 13 |

SD = standard deviation; **S** = saline; **NVP** = nevirapine; **LPS** = lipopolysaccharide; **Rx** = treatment; * rat died within 24 hours of LPS administration

APPENDIX E: LIVER FUNCTION TEST RESULTS

Appendix E-1: LFTs during the preliminary experiment

Table E-1: LFTs (u/l) of groups A (Saline), B (NVP low) and C (NVP high) over 28 days

| Group | LFT | Rat 1 | Rat 2 | Rat 3 | Rat 4 | Rat 5 | Mean | SD |
|---------------------|------------|-------|-------|-------|-------|-------|---------------|-------|
| A (saline) | | | | | | | | |
| 7 Days | ALP | 256 | 273 | 271 | - | 250 | 262.50 | 11.3 |
| | ALT | 106 | 60 | 58 | - | 55 | 69.75 | 24.3 |
| | AST | 330 | 114 | 113 | - | 120 | 169.25 | 107.2 |
| 14 Days | ALP | 244 | 232 | 275 | 372 | 246 | 273.80 | 57.1 |
| | ALT | 51 | 55 | 51 | 63 | 57 | 55.40 | 4.9 |
| | AST | 108 | 129 | 115 | 104 | 123 | 115.80 | 10.3 |
| 21 Days | ALP | 291 | 239 | 281 | 335 | 279 | 285.00 | 34.3 |
| | ALT | 67 | 57 | 56 | 59 | 50 | 57.80 | 6.1 |
| | AST | 112 | 128 | 138 | 126 | 100 | 120.80 | 14.9 |
| 28 Days | ALP | 233 | 329 | 285 | 271 | 291 | 281.80 | 34.7 |
| | ALT | 58 | 59 | 58 | 61 | 59 | 59.00 | 1.2 |
| | AST | 112 | 122 | 118 | 112 | 108 | 114.40 | 5.6 |
| B (NVP low) | | | | | | | | |
| 7 Days | ALP | 305 | 254 | 253 | 225 | 197 | 246.80 | 40.1 |
| | ALT | 61 | 65 | 65 | 55 | 52 | 59.60 | 5.9 |
| | AST | 112 | 143 | 99 | 124 | 105 | 115.60 | 17.4 |
| 14 Days | ALP | 252 | 208 | 272 | 244 | 258 | 246.80 | 23.9 |
| | ALT | 58 | 54 | 59 | 57 | 53 | 56.20 | 2.6 |
| | AST | 98 | 257 | 141 | 93 | 200 | 157.80 | 70.1 |
| 21 Days | ALP | 232 | 231 | 233 | 309 | 275 | 256.00 | 35.0 |
| | ALT | 56 | 54 | 65 | 58 | 52 | 57.00 | 5.0 |
| | AST | 149 | 129 | 116 | 126 | 93 | 122.60 | 20.4 |
| 28 Days | ALP | 213 | 222 | 213 | 306 | 270 | 244.80 | 41.6 |
| | ALT | 65 | 57 | 61 | 77 | 86 | 69.20 | 12.1 |
| | AST | 131 | 127 | 128 | 145 | 243 | 154.80 | 49.8 |
| C (NVP high) | | | | | | | | |
| 7 Days | ALP | 182 | 351 | 234 | 190 | 252 | 241.80 | 67.7 |
| | ALT | 53 | 60 | 49 | 52 | 66 | 56.00 | 6.9 |
| | AST | 94 | 102 | 80 | 79 | 83 | 87.60 | 10.1 |
| 14 Days | ALP | 266 | 263 | 243 | 225 | 185 | 236.40 | 33.2 |
| | ALT | 54 | 54 | 50 | 49 | 67 | 54.80 | 7.2 |
| | AST | 85 | 94 | 87 | 81 | 114 | 92.20 | 13.1 |
| 21 Days | ALP | 229 | 258 | 199 | 203 | 268 | 231.40 | 31.3 |
| | ALT | 54 | 54 | 58 | 55 | 56 | 55.40 | 1.7 |
| | AST | 140 | 89 | 79 | 136 | 82 | 105.20 | 30.2 |
| 28 Days | ALP | 270 | 233 | 290 | 183 | 303 | 255.80 | 48.5 |
| | ALT | 54 | 55 | 55 | 51 | 65 | 56.00 | 5.3 |
| | AST | 88 | 114 | 101 | 96 | 98 | 99.40 | 9.5 |

LFT = liver function test; SD = standard deviation; NVP = nevirapine; ALP = alkaline phosphatase; ALT = alanine aminotransferase; AST = aspartate aminotransferase

Appendix E-2: LFTs during the acute phase

Table E-2: LFTs (u/l) of groups D (S+NVP), E (LPS+S) and F (LPS+NVP) over 24 hours

| Group | LFT | Rat 1 | Rat 2 | Rat 3 | Rat 4 | Rat 5 | Mean | SD |
|--------------------|------------|-------|-------|-------|-------|-------|---------------|------|
| D (S+NVP) | | | | | | | | |
| 6 Hours | ALP | 292 | 310 | 300 | 244 | 194 | 268.00 | 48.5 |
| | ALT | 60 | 57 | 54 | 44 | 51 | 53.20 | 6.1 |
| | AST | 108 | 101 | 97 | 104 | 247 | 131.40 | 64.8 |
| 24 Hours | ALP | 219 | 281 | 248 | 310 | 300 | 271.60 | 37.7 |
| | ALT | 53 | 70 | 68 | 73 | 72 | 67.20 | 8.2 |
| | AST | 179 | 121 | 140 | 130 | 105 | 135.00 | 27.8 |
| E (LPS+S) | | | | | | | | |
| 6 Hours | ALP | 213 | 267 | 184 | 195 | 212 | 214.20 | 31.9 |
| | ALT | 127 | 57 | 60 | 90 | 82 | 83.20 | 28.2 |
| | AST | 190 | 127 | 160 | 159 | 169 | 161.00 | 22.7 |
| 24 Hours | ALP | 221 | 289 | 287 | 263 | 314 | 274.80 | 35.1 |
| | ALT | 33 | 30 | 26 | 29 | 34 | 30.40 | 3.2 |
| | AST | 228 | 122 | 127 | 80 | 100 | 131.40 | 57.2 |
| F (LPS+NVP) | | | | | | | | |
| 6 Hours | ALP | 231 | 157 | 158 | 160 | 172 | 175.60 | 31.6 |
| | ALT | 47 | 86 | 65 | 51 | 56 | 61.00 | 15.5 |
| | AST | 104 | 112 | 123 | 98 | 234 | 134.20 | 56.6 |
| 24 Hours | ALP | 191 | 222 | 200 | 237 | 179 | 205.80 | 23.5 |
| | ALT | 32 | 39 | 24 | 38 | 22 | 31.00 | 7.8 |
| | AST | 134 | 101 | 108 | 112 | 78 | 106.60 | 20.2 |

LFT = liver function test; SD = standard deviation; S = saline; NVP = nevirapine; LPS = lipopolysaccharide; ALP = alkaline phosphatase; ALT = alanine aminotransferase; AST = aspartate aminotransferase;

Appendix E-3: LFTs during the chronic phase

Table E-3: LFTs (u/l) of groups G (S+NVP) and H (LPS+NVP) over 21 days

| Group | LFT | Rat 1 | Rat 2 | Rat 3 | Rat 4 | Rat 5 | Mean | SD |
|--------------------|------------|-------|-------|-------|-------|-------|---------------|-------|
| G (S+NVP) | | | | | | | | |
| 7 Days | ALP | 200 | 168 | 175 | 236 | - | 194.75 | 30.7 |
| | ALT | 65 | 48 | 53 | 68 | - | 58.50 | 9.5 |
| | AST | 127 | 224 | 103 | 102 | - | 139.00 | 57.8 |
| 14 Days | ALP | 179 | 206 | 241 | - | - | 208.67 | 31.1 |
| | ALT | 74 | 82 | 70 | - | - | 75.33 | 6.1 |
| | AST | 112 | 155 | 111 | - | - | 126.00 | 25.1 |
| 21 Days | ALP | 205 | 170 | 81 | - | - | 152.00 | 63.9 |
| | ALT | 56 | 49 | 42 | - | - | 49.00 | 7.0 |
| | AST | 117 | 99 | 121 | - | - | 112.33 | 11.7 |
| H (LPS+NVP) | | | | | | | | |
| 7 Days | ALP | 376 | 246 | 189 | 802 | -* | 403.25 | 277.1 |
| | ALT | 88 | 93 | 65 | 342 | -* | 147.00 | 130.6 |
| | AST | 307 | 219 | 157 | 1025 | -* | 427.00 | 403.4 |
| 14 Days | ALP | 147 | 137 | 167 | 147 | 155 | 150.60 | 11.2 |
| | ALT | 70 | 56 | 49 | 43 | 42 | 52.00 | 11.5 |
| | AST | 143 | 133 | 108 | 89 | 86 | 111.80 | 25.6 |
| 21 Days | ALP | 172 | 182 | 161 | 151 | 159 | 165.00 | 12.1 |
| | ALT | 29 | 42 | 30 | 40 | 28 | 33.80 | 6.7 |
| | AST | 83 | 109 | 79 | 100 | 78 | 89.80 | 13.9 |

LFT = liver function test; SD = standard deviation; S = saline; NVP = nevirapine; LPS = lipopolysaccharide; ALP = alkaline phosphatase; ALT = alanine aminotransferase; AST = aspartate aminotransferase; * rat died within 24 hours of LPS administration

APPENDIX F: IMMUNOLOGY TEST RESULTS

Appendix F-1: Serum IL-2 levels of rats during the acute and chronic phases

Table F-1: Serum IL-2 levels (pg/ml) of untreated rats and groups D (S+NVP), E (LPS+S), F (LPS+NVP), G (S+NVP) and H (LPS+NVP)

| Group | Rat 1 | Rat 2 | Rat 3 | Rat 4 | Rat 5 | Mean | Median |
|--------------------|--------|--------|--------|--------|--------|--------------|--------------------|
| Untreated | | | | | | | |
| 0 Hours | 254.91 | 243.56 | 218.01 | - | - | 238.83±18.9 | 243.6(336.2-472.9) |
| D (S+NVP) | | | | | | | |
| 6 Hours | 421.63 | 381.08 | 355.39 | - | - | 386.02±33.4 | 381.0(340.4-434.5) |
| 24 Hours | 363.94 | 387.44 | 355.39 | - | - | 368.92±16.6 | 363.9(340.4-404.5) |
| E (LPS+S) | | | | | | | |
| 6 Hours | 271.94 | 393.99 | 379.80 | 286.13 | 254.91 | 317.36±64.6 | 291.8(319.1-592.6) |
| 24 Hours | 561.45 | 348.58 | 643.76 | - | - | 517.93±152.3 | 391.2(336.2-472.9) |
| F (LPS+NVP) | | | | | | | |
| 6 Hours | 372.48 | 483.60 | 475.05 | 443.00 | 334.02 | 421.63±65.7 | 372.5(319.1-592.6) |
| 24 Hours | 395.99 | 351.12 | 363.94 | 453.68 | 428.04 | 398.55±42.9 | 387.4(336.2-472.9) |
| G (S+NVP) | | | | | | | |
| 7 Days | 453.68 | 321.20 | 329.75 | 334.02 | - | 359.66±62.3 | 331.9(310.5-498.6) |
| 14 Days | 387.44 | 314.79 | 434.48 | - | - | 378.90±60.3 | 387.4(301.9-434.5) |
| 21 Days | 310.52 | 509.24 | 588.30 | - | - | 469.35±143.1 | 413.1(280.6-690.9) |
| H (LPS+NVP) | | | | | | | |
| 7 Days | 284.88 | 308.38 | 306.24 | 295.56 | -* | 298.76±10.8 | 304.1(267.8-314.8) |
| 14 Days | 374.62 | 410.95 | 336.16 | 334.02 | 304.11 | 351.97±41.4 | 334.0(293.4-494.3) |
| 21 Days | 314.79 | 314.79 | 348.98 | 299.83 | 287.01 | 313.08±23.2 | 310.5(273.3-366.1) |

S = saline; **NVP** = nevirapine; **LPS** = lipopolysaccharide; * rat died within 24 hours of LPS administration

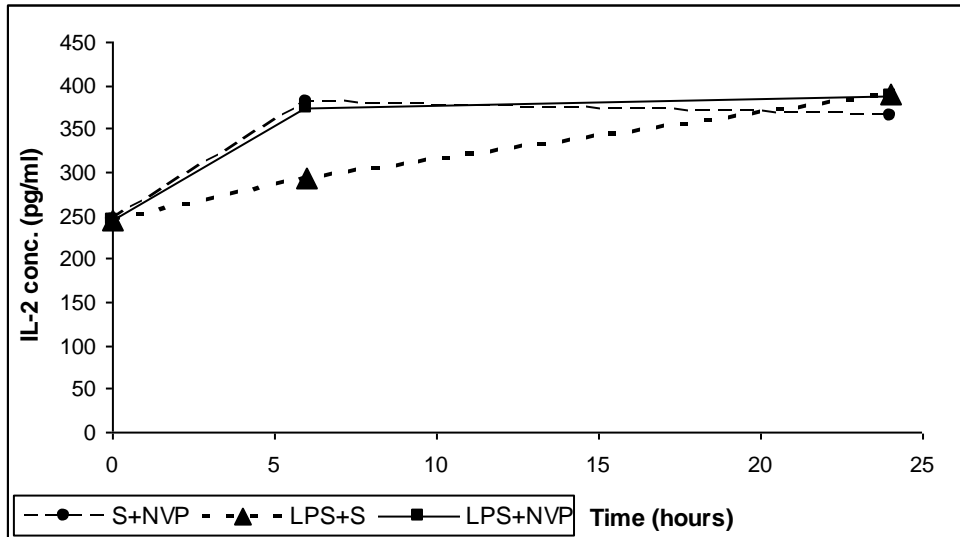


Figure F-1: Median values of serum IL-2 levels of groups D (S+NVP), E (LPS+S) and F (LPS+NVP) at 6 and 24 hours after dosing

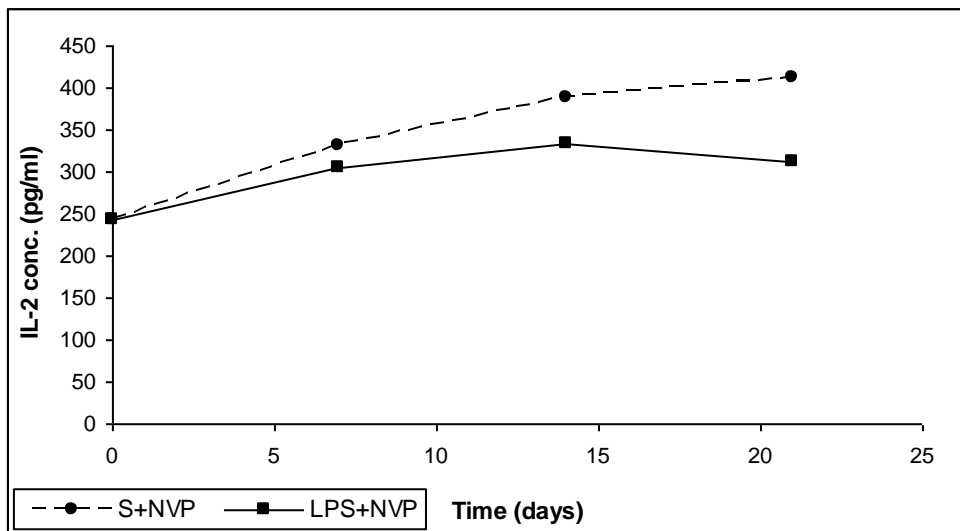


Figure F-2: Median values of serum IL-2 levels of groups G (S+NVP) and H (LPS+NVP) over a 21 day dosing period

Appendix F-2: Serum IFN- γ levels of rats during the acute and chronic phases

Table F-2: Serum IFN- γ levels (pg/ml) of untreated rats and groups D (S+NVP), E (LPS+S), F (LPS+NVP), G (S+NVP) and H (LPS+NVP)

| Group | Rat 1 | Rat 2 | Rat 3 | Rat 4 | Rat 5 | Mean | Median |
|--------------------|--------|--------|--------|--------|--------|-------------------|--------------------|
| Untreated | | | | | | | |
| 0 Hours | 130.50 | 138.33 | 147.12 | - | - | 138.65 \pm 8.3 | 134.4(336.2-472.9) |
| D (S+NVP) | | | | | | | |
| 6 Hours | 244.06 | 126.62 | 105.48 | - | - | 158.72 \pm 74.7 | 136.0(89.1-276.9) |
| 24 Hours | 112.53 | 145.41 | 84.34 | - | - | 114.09 \pm 30.6 | 112.5(70.2-154.8) |
| E (LPS+S) | | | | | | | |
| 6 Hours | 190.15 | 139.30 | 196.01 | 135.39 | 140.28 | 160.23 \pm 30.1 | 151.0(319.1-592.6) |
| 24 Hours | 143.21 | 140.28 | 108.02 | - | - | 130.50 \pm 19.5 | 123.7(336.2-472.9) |
| F (LPS+NVP) | | | | | | | |
| 6 Hours | 112.53 | 96.09 | 265.20 | 112.53 | 117.23 | 140.71 \pm 70.1 | 117.2(89.1-276.9) |
| 24 Hours | 110.18 | 100.78 | 133.67 | 150.11 | 121.92 | 123.33 \pm 19.4 | 129.0(112.5-159.5) |
| G (S+NVP) | | | | | | | |
| 7 Days | 199.57 | 133.67 | 91.39 | 140.71 | - | 121.34 \pm 21.8 | 119.6(79.6-173.6) |
| 14 Days | 100.78 | 126.62 | 157.16 | - | - | 128.19 \pm 28.2 | 126.6(84.3-178.3) |
| 21 Days | 234.67 | 131.32 | 136.02 | - | - | 167.33 \pm 58.4 | 133.7(103.1-220.6) |
| H (LPS+NVP) | | | | | | | |
| 7 Days | 86.69 | 81.99 | 89.04 | 154.81 | -* | 103.13 \pm 34.6 | 91.4(70.2-178.3) |
| 14 Days | 150.11 | 114.88 | 98.44 | 107.83 | 74.95 | 109.24 \pm 27.4 | 107.8(70.2-164.2) |
| 21 Days | 121.92 | 150.11 | 124.27 | 112.53 | 112.53 | 124.27 \pm 15.4 | 112.5(74.9-220.6) |

S = saline; **NVP** = nevirapine; **LPS** = lipopolysaccharide; * rat died within 24 hours of LPS administration

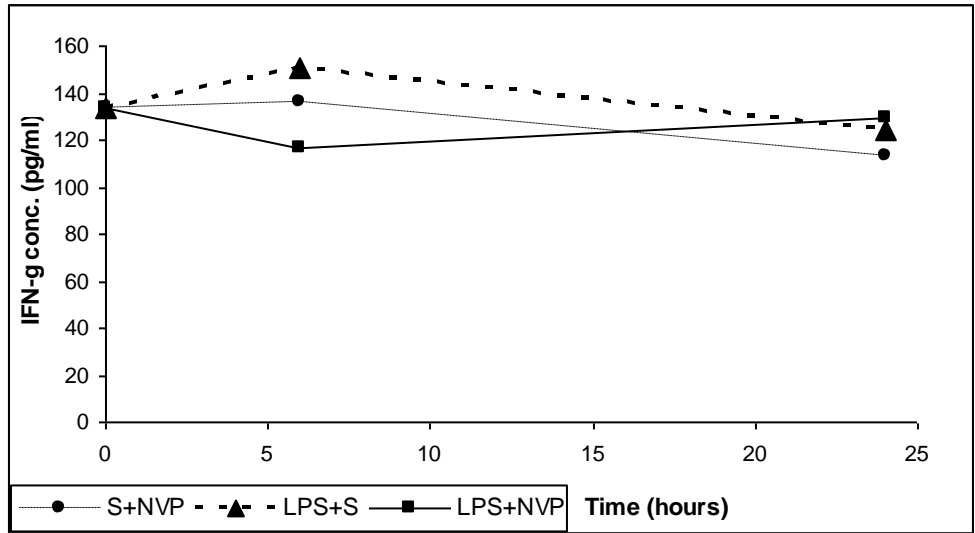


Figure F-3: Median values of serum IFN- γ levels of groups D (S+NVP), E (LPS+S) and F (LPS+NVP) at 6 and 24 hours after dosing

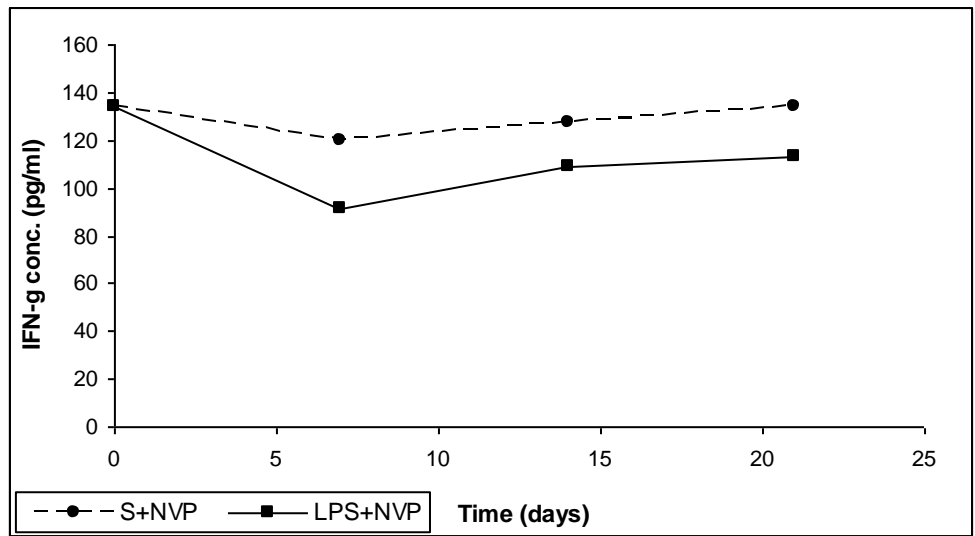


Figure F-4: Median values of serum IFN- γ levels of groups G (S+NVP) and H (LPS+NVP) over a 21 day dosing period

Appendix F-3: Serum TNF- α levels of rats during the acute and chronic phases

Table F-3: Serum TNF- α levels (pg/ml) of untreated rats and groups D (S+NVP), E (LPS+S), F (LPS+NVP), G (S+NVP) and H (LPS+NVP)

| Group | Rat 1 | Rat 2 | Rat 3 | Rat 4 | Rat 5 | Mean | Median |
|--------------------|----------|---------|---------|----------|---------|----------------------|-----------------------|
| Untreated | | | | | | | |
| 0 Hours | 62.68 | 150.34 | 97.26 | - | - | 103.43 \pm 44.2 | 97.3(35.3-178.0) |
| D (S+NVP) | | | | | | | |
| 6 Hours | 604.10 | 934.08 | 1084.08 | - | - | 874.09 \pm 245.5 | 732.2(564.1-1454.1) |
| 24 Hours | 3833.95 | 3368.97 | 3598.96 | - | - | 3600.63 \pm 232.5 | 3840.4(234.1-6503.8) |
| E (LPS+S) | | | | | | | |
| 6 Hours | 41.26 | 37.29 | 41.05 | 40.42 | 122.13 | 56.43 \pm 42.1 | 41.0(19.4-217.7) |
| 24 Hours | 20.47 | 14.62 | 89.84 | - | - | 41.64 \pm 41.8 | 121.0(634.1-4113.9) |
| F (LPS+NVP) | | | | | | | |
| 6 Hours | 489.60 | 1409.06 | 799.09 | 2189.21 | 3363.97 | 1650.19 \pm 1157.3 | 1379.2(144.1-5473.9) |
| 24 Hours | 954.08 | 154.12 | 729.09 | 409.11 | 994.08 | 648.10 \pm 360.9 | 464.8(124.1-1734.1) |
| G (S+NVP) | | | | | | | |
| 7 Days | 219.12 | 1224.07 | 794.09 | 11953.58 | - | 3547.71 \pm 5619.0 | 814.1(104.1-13963.5) |
| 14 Days | 1579.05 | 1624.05 | 514.10 | - | - | 1239.07 \pm 628.2 | 1234.1(264.1-2014.0) |
| 21 Days | 739.09 | 919.08 | 649.10 | - | - | 769.09 \pm 137.5 | 649.1(244.1-11393.6) |
| H (LPS+NVP) | | | | | | | |
| 7 Days | 10628.64 | 3158.98 | 2414.02 | 5413.88 | -* | 5403.88 \pm 3709.3 | 3094.0(424.1-19873.2) |
| 14 Days | 804.09 | 6098.85 | 2504.01 | 824.09 | 1124.08 | 2271.02 \pm 2251.0 | 1179.1(344.1-9563.7) |
| 21 Days | 264.11 | 599.10 | 2354.02 | 294.11 | 5888.86 | 1880.04 \pm 2401.2 | 379.1(244.1-11393.6) |

S = saline; NVP = nevirapine; LPS = lipopolysaccharide; * rat died within 24 hours of LPS administration

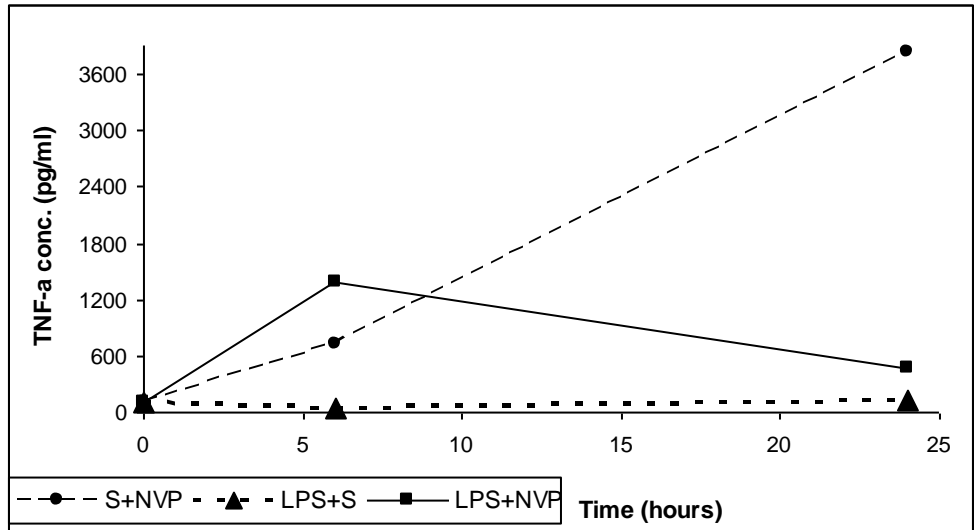


Figure F-5: Median values of serum TNF- α levels of groups D (S+NVP), E (LPS+S) and F (LPS+NVP) at 6 and 24 hours after dosing

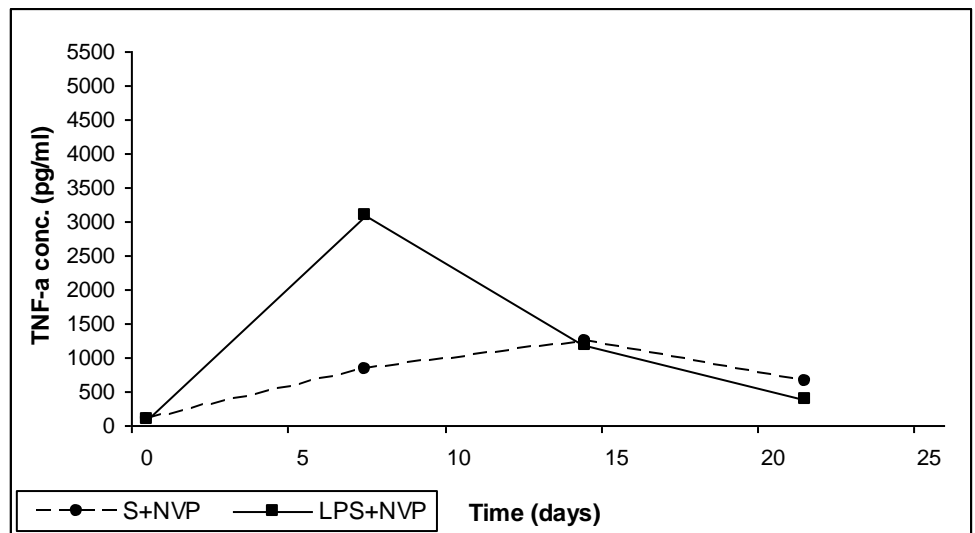


Figure F-6: Median values of serum TNF- α levels of groups G (S+NVP) and H (LPS+NVP) over a 21 day dosing period

APPENDIX G: FULL BLOOD COUNT RESULTS

Appendix G-1: FBC during the chronic phase

Table G-1: FBC of groups G (S+NVP) and H (LPS+NVP) after 21 days of dosing

| Group | Rat 1 | Rat 2 | Rat 3 | Rat 4 | Rat 5 | Mean | SD |
|--------------------|-------|-------|-------|-------|-------|---------------|-------|
| G (S+NVP) | | | | | | | |
| Red cell count | 8.60 | 8.76 | 9.38 | - | - | 8.91 | 0.4 |
| Haemoglobin | 16.20 | 16.40 | 17.50 | - | - | 16.70 | 0.7 |
| Haematocrit | 0.72 | 0.73 | 0.77 | - | - | 0.74 | 0.0 |
| MCV | 84 | 84 | 82 | - | - | 83.33 | 1.2 |
| MCH | 19 | 19 | 19 | - | - | 19 | 0.0 |
| MCHC | 22 | 22 | 23 | - | - | 22.3 | 0.6 |
| White cell count | 11.50 | 10.50 | 10.70 | - | - | 10.90 | 0.5 |
| Neutrophils | 1.15 | 2.30 | 3.21 | - | - | 2.22 | 1.0 |
| Lymphocytes | 9.55 | 7.46 | 6.85 | - | - | 7.95 | 1.4 |
| Monocytes | 0.69 | 0.53 | 0.54 | - | - | 0.59 | 0.1 |
| Eosinophils | 0.12 | 0.07 | 0.11 | - | - | 0.10 | 0.0 |
| Basophils | 0.00 | 0.10 | 0.00 | - | - | 0.10 | 0.0 |
| Platelet count | 794 | 968 | 1162 | - | - | 974.67 | 184.1 |
| H (LPS+NVP) | | | | | | | |
| Red cell count | 8.93 | 8.94 | 9.11 | 9.05 | 9.07 | 9.08 | 0.1 |
| Haemoglobin | 15.90 | 16.60 | 16.90 | 16.80 | 17.10 | 16.93 | 0.5 |
| Haematocrit | 0.73 | 0.75 | 0.70 | 0.74 | 0.75 | 0.73 | 0.0 |
| MCV | 82 | 84 | 84 | 82 | 83 | 83.00 | 1.0 |
| MCH | 18 | 19 | 19 | 19 | 19 | 19.00 | 0.5 |
| MCHC | 22 | 22 | 22 | 23 | 23 | 22.70 | 0.6 |
| White cell count | 11.50 | 15.80 | 16.50 | 13.60 | 15.90 | 15.33 | 2.1 |
| Neutrophils | 6.33 | 7.90 | 9.41 | 7.89 | 9.38 | 8.89 | 1.3 |
| Lymphocytes | 4.37 | 7.27 | 5.12 | 5.30 | 5.57 | 5.33 | 1.1 |
| Monocytes | 0.69 | 0.63 | 0.99 | 0.27 | 0.95 | 0.74 | 0.5 |
| Eosinophils | 0.12 | 0.00 | 0.99 | 0.14 | 0.00 | 0.57 | 0.5 |
| Basophils | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.0 |
| Platelet count | 444 | 648 | 656 | 584 | 563 | 601.00 | 85.4 |

SD = standard deviation; S = saline; NVP = nevirapine; LPS = lipopolysaccharide; MCV = mean corpuscular volume; MCH = mean corpuscular haemoglobin; MCHC = mean corpuscular haemoglobin concentration

APPENDIX H: NEVIRAPINE PLASMA MONITORING

Appendix H-1: Nevirapine plasma levels of all groups

Table H-1: Nevirapine plasma levels ($\mu\text{g/ml}$) of groups B (NVP low), C (NVP high), D (S+NVP), F (LPS+NVP), G (S+NVP) and H (LPS+NVP)

| Group | Rat 1 | Rat 2 | Rat 3 | Rat 4 | Rat 5 | Mean | SD |
|---------------------|-------|-------|-------|-------|-------|-------------|-----|
| B (NVP low) | | | | | | | |
| 7 Days | 1.15 | 0.40 | 0.00 | 1.41 | 0.00 | 0.59 | 0.7 |
| 14 Days | 0.12 | 0.15 | 0.23 | 0.08 | 0.26 | 0.17 | 0.1 |
| 21 Days | 0.19 | 0.00 | 0.38 | 0.20 | 0.27 | 0.21 | 0.1 |
| 28 Days | 0.52 | 0.30 | 0.25 | 0.23 | 0.23 | 0.30 | 0.1 |
| C (NVP high) | | | | | | | |
| 7 Days | 0.19 | 0.12 | 0.15 | 0.17 | 0.13 | 0.15 | 0.0 |
| 14 Days | 0.00 | 0.15 | 0.00 | 0.00 | 0.00 | 0.03 | 0.1 |
| 21 Days | 0.00 | 0.00 | 0.00 | 0.00 | 0.18 | 0.04 | 0.1 |
| 28 Days | 0.20 | 0.26 | 0.26 | 0.19 | 0.22 | 0.23 | 0.0 |
| D (S+NVP) | | | | | | | |
| 6 Hours | 0.64 | 1.57 | 5.67 | 2.56 | 7.80 | 3.65 | 3.0 |
| 24 Hours | 0.92 | 0.30 | 0.32 | 0.79 | 0.14 | 0.49 | 0.3 |
| F (LPS+NVP) | | | | | | | |
| 6 Hours | 3.71 | 0.55 | 0.98 | 0.63 | 0.66 | 1.31 | 1.4 |
| 24 Hours | 1.12 | 3.25 | 2.46 | 3.02 | 2.17 | 2.40 | 0.8 |
| G (S+NVP) | | | | | | | |
| 7 Days | 0.65 | 1.21 | 0.38 | 0.75 | - | 0.75 | 0.4 |
| 14 Days | 0.11 | 0.12 | 0.09 | - | - | 0.10 | 0.0 |
| 21 Days | 0.07 | 0.06 | 0.04 | - | - | 0.06 | 0.0 |
| H (LPS+NVP) | | | | | | | |
| 7 Days | 1.72 | 0.59 | 1.06 | 0.15 | -* | 0.88 | 0.6 |
| 14 Days | 0.07 | 0.37 | 0.41 | 0.63 | 0.91 | 0.48 | 0.3 |
| 21 Days | 0.62 | 1.26 | 0.66 | 1.09 | 0.69 | 0.86 | 0.3 |

SD = standard deviation; NVP = nevirapine; S = saline; LPS = lipopolysaccharide; * rat died within 24 hours of LPS administration

APPENDIX I: P-VALUES

Appendix I-1: P-values of rat weights of untreated rats and groups G (S+NVP) and H (LPS+NVP) during the chronic phase

Kruskal-Wallis Test (Nonparametric ANOVA)

The P value is <0.0001, considered extremely significant. Variation among column medians is significantly greater than expected by chance.

The P value is approximate (from chi-square distribution) because at least one column has two or more identical values.

Kruskal-Wallis Statistic KW = 67.505 (corrected for ties)

Dunn's Multiple Comparisons Test

| Comparison | Mean Rank Difference | P value |
|----------------------------------|----------------------|-----------|
| UnRx 7d Rx0 vs. UnRx 7d Rx7 | -6.400 | ns P>0.05 |
| UnRx 7d Rx0 vs. UnRx 14d Rx0 | -2.600 | ns P>0.05 |
| UnRx 7d Rx0 vs. UnRx 21d Rx0 | 9.200 | ns P>0.05 |
| UnRx 7d Rx0 vs. S+NVP 7d Rx0 | 49.650 | ns P>0.05 |
| UnRx 7d Rx0 vs. S+NVP 7d Rx7 | 37.400 | ns P>0.05 |
| UnRx 7d Rx0 vs. LPS+N 7d Rx0 | 39.500 | ns P>0.05 |
| UnRx 7d Rx7 vs. UnRx 14d Rx14 | -4.100 | ns P>0.05 |
| UnRx 7d Rx7 vs. UnRx 21d Rx21 | 4.100 | ns P>0.05 |
| UnRx 7d Rx7 vs. S+NVP 7d Rx7 | 43.800 | ns P>0.05 |
| UnRx 7d Rx7 vs. LPS+N 7d Rx7 | 56.400 | * P<0.05 |
| UnRx 14d Rx0 vs. UnRx 14d Rx14 | -7.900 | ns P>0.05 |
| UnRx 14d Rx0 vs. UnRx 21d Rx0 | 11.800 | ns P>0.05 |
| UnRx 14d Rx0 vs. S+NVP 14d Rx0 | 55.333 | ns P>0.05 |
| UnRx 14d Rx0 vs. LPS+N 14d Rx0 | 46.700 | ns P>0.05 |
| UnRx 14d Rx14 vs. UnRx 21d Rx21 | 8.200 | ns P>0.05 |
| UnRx 14d Rx14 vs. S+NVP 14d Rx14 | 46.567 | ns P>0.05 |
| UnRx 14d Rx14 vs. LPS+N 14d Rx14 | 43.200 | ns P>0.05 |
| UnRx 21d Rx0 vs. UnRx 21d Rx21 | -11.500 | ns P>0.05 |
| UnRx 21d Rx0 vs. S+NVP 21 d Rx0 | 24.533 | ns P>0.05 |

| | | | |
|-----------------------------------|---------|----|--------|
| UnRx 21d Rx0 vs. LPS+N 21d Rx0 | 30.200 | ns | P>0.05 |
| UnRx 21d Rx21 vs. S+NVP 7d Rx0 | 51.950 | ns | P>0.05 |
| UnRx 21d Rx21 vs. S+NVP 21d Rx21 | 18.033 | ns | P>0.05 |
| UnRx 21d Rx21 vs. LPS+N 21d Rx21 | 23.000 | ns | P>0.05 |
| S+NVP 7d Rx0 vs. S+NVP 7d Rx7 | -12.250 | ns | P>0.05 |
| S+NVP 7d Rx0 vs. S+NVP 14d Rx0 | 3.083 | ns | P>0.05 |
| S+NVP 7d Rx0 vs. S+NVP 21 d Rx0 | -15.917 | ns | P>0.05 |
| S+NVP 7d Rx0 vs. LPS+N 7d Rx0 | -10.150 | ns | P>0.05 |
| S+NVP 7d Rx7 vs. S+NVP 14d Rx14 | -1.333 | ns | P>0.05 |
| S+NVP 7d Rx7 vs. S+NVP 21d Rx21 | -21.667 | ns | P>0.05 |
| S+NVP 7d Rx7 vs. LPS+N 7d Rx7 | 12.600 | ns | P>0.05 |
| S+NVP 14d Rx0 vs. S+NVP 14d Rx14 | -16.667 | ns | P>0.05 |
| S+NVP 14d Rx0 vs. S+NVP 21d Rx0 | -19.000 | ns | P>0.05 |
| S+NVP 14d Rx0 vs. LPS+N 14d Rx0 | -8.633 | ns | P>0.05 |
| S+NVP 14d Rx14 vs. S+NVP 21d Rx21 | -20.333 | ns | P>0.05 |
| S+NVP 14d Rx14 vs. LPS+N 14d Rx14 | -3.367 | ns | P>0.05 |
| S+NVP 21 d Rx0 vs. S+NVP 21d Rx21 | -18.000 | ns | P>0.05 |
| S+NVP 21 d Rx0 vs. LPS+N 21d Rx0 | 5.667 | ns | P>0.05 |
| S+NVP 21d Rx21 vs. LPS+N 21d Rx21 | 4.967 | ns | P>0.05 |
| LPS+N 7d Rx0 vs. LPS+N 7d Rx7 | 10.500 | ns | P>0.05 |
| LPS+N 7d Rx0 vs. LPS+N 14d Rx0 | 4.600 | ns | P>0.05 |
| LPS+N 7d Rx0 vs. LPS+N 21d Rx0 | -0.1000 | ns | P>0.05 |
| LPS+N 7d Rx7 vs. LPS+N 14d Rx14 | -17.300 | ns | P>0.05 |
| LPS+N 7d Rx7 vs. LPS+N 21d Rx21 | -29.300 | ns | P>0.05 |
| LPS+N 14d Rx0 vs. LPS+N 14d Rx14 | -11.400 | ns | P>0.05 |
| LPS+N 14d Rx0 vs. LPS+N 21d Rx0 | -4.700 | ns | P>0.05 |
| LPS+N 14d Rx14 vs. LPS+N 21d Rx21 | -12.000 | ns | P>0.05 |
| LPS+N 21d Rx0 vs. LPS+N 21d Rx21 | -18.700 | ns | P>0.05 |

Appendix I-2: P-values of LFTs of untreated rats and groups D (S+NVP), E (LPS+S) and F (LPS+NVP) during the acute phase

Alanine transaminase (ALT)

Kruskal-Wallis Test (Nonparametric ANOVA)

The P value is 0.0004, considered extremely significant. Variation among column medians is significantly greater than expected by chance.

The P value is approximate (from chi-square distribution) because at least one column has two or more identical values.

Kruskal-Wallis Statistic KW = 24.410 (corrected for ties)

Dunn's Multiple Comparisons Test

| Comparison | Mean Rank | |
|----------------------------|------------|-----------|
| | Difference | P value |
| Untreated vs. S+NVP 6h | 6.900 | ns P>0.05 |
| Untreated vs. LPS+S 6h | -4.200 | ns P>0.05 |
| Untreated vs. LPS+NVP 6h | 3.600 | ns P>0.05 |
| Untreated vs. S+NVP 24h | -1.500 | ns P>0.05 |
| Untreated vs. LPS+S 24h | 18.100 | ns P>0.05 |
| Untreated vs. LPS+NVP 24h | 17.900 | ns P>0.05 |
| S+NVP 6h vs. LPS+S 6h | -11.100 | ns P>0.05 |
| S+NVP 6h vs. LPS+NVP 6h | -3.300 | ns P>0.05 |
| S+NVP 6h vs. S+NVP 24h | -8.400 | ns P>0.05 |
| LPS+S 6h vs. LPS+NVP 6h | 7.800 | ns P>0.05 |
| LPS+S 6h vs. LPS+S 24h | 22.300 | ** P<0.01 |
| LPS+NVP 6h vs. LPS+NVP 24h | 14.300 | ns P>0.05 |
| S+NVP 24h vs. LPS+S 24h | 19.600 | * P<0.05 |
| S+NVP 24h vs. LPS+NVP 24h | 19.400 | * P<0.05 |
| LPS+S 24h vs. LPS+NVP 24h | -0.2000 | ns P>0.05 |

Appendix I-3: P-values of LFTs of untreated rats and groups G (S+NVP) and H (LPS+NVP) during the chronic phase

Alanine transaminase (ALT)

Kruskal-Wallis Test (Nonparametric ANOVA)

The P value is 0.0012, considered very significant. Variation among column medians is significantly greater than expected by chance.

The P value is approximate (from chi-square distribution) because at least one column has two or more identical values.

Kruskal-Wallis Statistic KW = 25.604 (corrected for ties)

Dunn's Multiple Comparisons Test

| Comparison | Mean Rank Difference | P value |
|-------------------------------------|----------------------|-----------|
| Untreated 7days vs. S+NVP 7days | 5.000 | ns P>0.05 |
| Untreated 7days vs. LPS+NVP 7days | -8.750 | ns P>0.05 |
| Untreated 7days vs. Untreated 14day | 7.175 | ns P>0.05 |
| Untreated 7days vs. Untreated 21day | 4.275 | ns P>0.05 |
| S+NVP 7days vs. LPS+NVP 7days | -13.750 | ns P>0.05 |
| S+NVP 7days vs. S+NVP 14days | -12.458 | ns P>0.05 |
| S+NVP 7days vs. S+NVP 21days | 8.542 | ns P>0.05 |
| LPS+NVP 7days vs. LPS+NVP 14days | 19.125 | ns P>0.05 |
| LPS+NVP 7days vs. LPS+NVP 21days | 30.925 | ** P<0.01 |
| Untreated 14day vs. S+NVP 14days | -14.633 | ns P>0.05 |
| Untreated 14day vs. LPS+NVP 14days | 3.200 | ns P>0.05 |
| Untreated 14day vs. Untreated 21day | -2.900 | ns P>0.05 |
| S+NVP 14days vs. LPS+NVP 14days | 17.833 | ns P>0.05 |
| S+NVP 14days vs. S+NVP 21days | 21.000 | ns P>0.05 |
| LPS+NVP 14days vs. LPS+NVP 21days | 11.800 | ns P>0.05 |
| Untreated 21day vs. S+NVP 21days | 9.267 | ns P>0.05 |
| Untreated 21day vs. LPS+NVP 21days | 17.900 | ns P>0.05 |
| S+NVP 21days vs. LPS+NVP 21days | 8.633 | ns P>0.05 |

Appendix I-4: P-values of cytokine levels of untreated rats and groups D (S+NVP), E (LPS+S) and F (LPS+NVP) during the acute phase

Interleukin-2 (IL-2)

Kruskal-Wallis Test (Nonparametric ANOVA)

The P value is 0.0011, considered very significant. Variation among column medians is significantly greater than expected by chance.

The P value is approximate (from chi-square distribution) because at least one column has two or more identical values.

Kruskal-Wallis Statistic KW = 22.172 (corrected for ties)

Dunn's Multiple Comparisons Test

| Comparison | Mean Rank Difference | P value |
|----------------------------|----------------------|-----------|
| Untreated vs. S+NVP 6h | -29.500 | * P<0.05 |
| Untreated vs. LPS+S 6h | -13.967 | ns P>0.05 |
| Untreated vs. LPS+NVP 6h | -29.767 | ** P<0.01 |
| Untreated vs. S+NVP 24h | -24.167 | ns P>0.05 |
| Untreated vs. LPS+S 24h | -28.583 | * P<0.05 |
| Untreated vs. LPS+NVP 24h | -30.667 | ** P<0.01 |
| S+NVP 6h vs. LPS+S 6h | 15.533 | ns P>0.05 |
| S+NVP 6h vs. LPS+NVP 6h | -0.2667 | ns P>0.05 |
| S+NVP 6h vs. S+NVP 24h | 5.333 | ns P>0.05 |
| LPS+S 6h vs. LPS+NVP 6h | -15.800 | ns P>0.05 |
| LPS+S 6h vs. LPS+S 24h | -14.617 | ns P>0.05 |
| LPS+NVP 6h vs. LPS+NVP 24h | -0.9000 | ns P>0.05 |
| S+NVP 24h vs. LPS+S 24h | -4.417 | ns P>0.05 |
| S+NVP 24h vs. LPS+NVP 24h | -6.500 | ns P>0.05 |
| LPS+S 24h vs. LPS+NVP 24h | -2.083 | ns P>0.05 |

Interferon-gamma (IFN-γ)

Kruskal-Wallis Test (Nonparametric ANOVA)

The P value is 0.1814, considered not significant.
Variation among column medians is not significantly
greater than expected by chance.

The P value is approximate (from chi-square distribution)
because at least one column has two or more identical
values.

Kruskal-Wallis Statistic KW = 8.864 (corrected for ties)

Dunn's Multiple Comparisons Test

| Comparison | Mean Rank Difference | P value |
|----------------------------|-------------------------|-----------|
| Untreated vs. S+NVP 6h | 2.917 | ns P>0.05 |
| Untreated vs. LPS+S 6h | -6.617 | ns P>0.05 |
| Untreated vs. LPS+NVP 6h | 9.533 | ns P>0.05 |
| Untreated vs. S+NVP 24h | 13.167 | ns P>0.05 |
| Untreated vs. LPS+S 24h | 4.917 | ns P>0.05 |
| Untreated vs. LPS+NVP 24h | 7.883 | ns P>0.05 |
| S+NVP 6h vs. LPS+S 6h | -9.533 | ns P>0.05 |
| S+NVP 6h vs. LPS+NVP 6h | 6.617 | ns P>0.05 |
| S+NVP 6h vs. S+NVP 24h | 10.250 | ns P>0.05 |
| LPS+S 6h vs. LPS+NVP 6h | 16.150 | ns P>0.05 |
| LPS+S 6h vs. LPS+S 24h | 11.533 | ns P>0.05 |
| LPS+NVP 6h vs. LPS+NVP 24h | -1.650 | ns P>0.05 |
| S+NVP 24h vs. LPS+S 24h | -8.250 | ns P>0.05 |
| S+NVP 24h vs. LPS+NVP 24h | -5.283 | ns P>0.05 |
| LPS+S 24h vs. LPS+NVP 24h | 2.967 | ns P>0.05 |

Tumour necrosis factor-alpha (TNF- α)

Kruskal-Wallis Test (Nonparametric ANOVA)

The P value is < 0.0001 , considered extremely significant.

Variation among column medians is significantly greater than expected by chance.

The P value is approximate (from chi-square distribution) because at least one column has two or more identical values.

Kruskal-Wallis Statistic KW = 40.936 (corrected for ties)

Dunn's Multiple Comparisons Test

| Comparison | Mean Rank Difference | P value |
|----------------------------|----------------------|-------------|
| Untreated vs. S+NVP 6h | -19.667 | ns P>0.05 |
| Untreated vs. LPS+S 6h | 5.867 | ns P>0.05 |
| Untreated vs. LPS+NVP 6h | -22.083 | ns P>0.05 |
| Untreated vs. S+NVP 24h | -30.083 | * P<0.05 |
| Untreated vs. LPS+S 24h | 9.500 | ns P>0.05 |
| Untreated vs. LPS+NVP 24h | -15.433 | ns P>0.05 |
| S+NVP 6h vs. LPS+S 6h | 25.533 | * P<0.05 |
| S+NVP 6h vs. LPS+NVP 6h | -2.417 | ns P>0.05 |
| S+NVP 6h vs. S+NVP 24h | -10.417 | ns P>0.05 |
| LPS+S 6h vs. LPS+NVP 6h | -27.950 | ** P<0.01 |
| LPS+S 6h vs. LPS+S 24h | 3.633 | ns P>0.05 |
| LPS+NVP 6h vs. LPS+NVP 24h | 6.650 | ns P>0.05 |
| S+NVP 24h vs. LPS+S 24h | 39.583 | *** P<0.001 |
| S+NVP 24h vs. LPS+NVP 24h | 14.650 | ns P>0.05 |
| LPS+S 24h vs. LPS+NVP 24h | -24.933 | * P<0.05 |

Appendix I-5: P-values of cytokine levels of untreated rats and groups G (S+NVP) and H (LPS+NVP) during the chronic phase

Interleukin-2 (IL-2)

Kruskal-Wallis Test (Nonparametric ANOVA)

The P value is < 0.0001, considered extremely significant.

Variation among column medians is significantly greater than expected by chance.

The P value is approximate (from chi-square distribution) because at least one column has two or more identical values.

Kruskal-Wallis Statistic KW = 28.115 (corrected for ties)

Dunn's Multiple Comparisons Test

| Comparison | Mean Rank Difference | P value |
|-----------------------------------|----------------------|-----------|
| Untreated vs. S+NVP 7days | -32.438 | ** P<0.01 |
| Untreated vs. LPS+NVP 7days | -14.125 | ns P>0.05 |
| Untreated vs. S+NVP 14days | -35.417 | ** P<0.01 |
| Untreated vs. LPS+NVP 14days | -29.750 | ** P<0.01 |
| Untreated vs. S+NVP 21days | -35.917 | ** P<0.01 |
| Untreated vs. LPS+NVP 21days | -19.800 | ns P>0.05 |
| S+NVP 7days vs. LPS+NVP 7days | 18.313 | ns P>0.05 |
| S+NVP 7days vs. S+NVP 14days | -2.979 | ns P>0.05 |
| S+NVP 7days vs. S+NVP 21days | -3.479 | ns P>0.05 |
| LPS+NVP 7days vs. LPS+NVP 14days | -15.625 | ns P>0.05 |
| LPS+NVP 7days vs. LPS+NVP 21days | -5.675 | ns P>0.05 |
| S+NVP 14days vs. LPS+NVP 14days | 5.667 | ns P>0.05 |
| S+NVP 14days vs. S+NVP 21days | -0.5000 | ns P>0.05 |
| LPS+NVP 14days vs. LPS+NVP 21days | 9.950 | ns P>0.05 |
| S+NVP 21days vs. LPS+NVP 21days | 16.117 | ns P>0.05 |

Interferon-gamma (IFN-γ)

Kruskal-Wallis Test (Nonparametric ANOVA)

The P value is 0.1777, considered not significant.
Variation among column medians is not significantly greater than expected by chance.

The P value is approximate (from chi-square distribution) because at least one column has two or more identical values.

Kruskal-Wallis Statistic KW = 8.927 (corrected for ties)

Dunn's Multiple Comparisons Test

| Comparison | Mean Rank Difference | P value |
|-----------------------------------|----------------------|-----------|
| Untreated vs. S+NVP 7days | 9.521 | ns P>0.05 |
| Untreated vs. LPS+NVP 7days | 19.333 | ns P>0.05 |
| Untreated vs. S+NVP 14days | 5.417 | ns P>0.05 |
| Untreated vs. LPS+NVP 14days | 15.383 | ns P>0.05 |
| Untreated vs. S+NVP 21days | 1.000 | ns P>0.05 |
| Untreated vs. LPS+NVP 21days | 10.783 | ns P>0.05 |
| S+NVP 7days vs. LPS+NVP 7days | 9.813 | ns P>0.05 |
| S+NVP 7days vs. S+NVP 14days | -4.104 | ns P>0.05 |
| S+NVP 7days vs. S+NVP 21days | -8.521 | ns P>0.05 |
| LPS+NVP 7days vs. LPS+NVP 14days | -3.950 | ns P>0.05 |
| LPS+NVP 7days vs. LPS+NVP 21days | -8.550 | ns P>0.05 |
| S+NVP 14days vs. LPS+NVP 14days | 9.967 | ns P>0.05 |
| S+NVP 14days vs. S+NVP 21days | -4.417 | ns P>0.05 |
| LPS+NVP 14days vs. LPS+NVP 21days | -4.600 | ns P>0.05 |
| S+NVP 21 days vs. LPS+NVP 21days | 9.783 | ns P>0.05 |

Tumour necrosis factor-alpha (TNF- α)

Kruskal-Wallis Test (Nonparametric ANOVA)

The P value is 0.0016, considered very significant.
Variation among column medians is significantly greater than expected by chance.

The P value is approximate (from chi-square distribution) because at least one column has two or more identical values.

Kruskal-Wallis Statistic KW = 21.376 (corrected for ties)

Dunn's Multiple Comparisons Test

| Comparison | Mean Rank | |
|-----------------------------------|------------|-------------|
| | Difference | P value |
| Untreated vs. S+NVP 7days | -23.104 | ns P>0.05 |
| Untreated vs. LPS+NVP 7days | -36.917 | *** P<0.001 |
| Untreated vs. S+NVP 14 days | -26.833 | ns P>0.05 |
| Untreated vs. LPS+NVP 14days | -28.767 | ** P<0.01 |
| Untreated vs. S+NVP 21days | -18.917 | ns P>0.05 |
| Untreated vs. LPS+NVP 21days | -20.867 | ns P>0.05 |
| S+NVP 7days vs. LPS+NVP 7days | -13.813 | ns P>0.05 |
| S+NVP 7days vs. S+NVP 14 days | -3.729 | ns P>0.05 |
| S+NVP 7days vs. S+NVP 21days | 4.188 | ns P>0.05 |
| LPS+NVP 7days vs. LPS+NVP 14days | 8.150 | ns P>0.05 |
| LPS+NVP 7days vs. LPS+NVP 21days | 16.050 | ns P>0.05 |
| S+NVP 14 days vs. LPS+NVP 14days | -1.933 | ns P>0.05 |
| S+NVP 14 days vs. S+NVP 21days | 7.917 | ns P>0.05 |
| LPS+NVP 14days vs. LPS+NVP 21days | 7.900 | ns P>0.05 |
| S+NVP 21days vs. LPS+NVP 21days | -1.950 | ns P>0.05 |

Appendix I-6: P-values of NVP plasma concentrations of groups D (S+NVP) and F (LPS+NVP) during the acute phase

Kruskal-Wallis Test (Nonparametric ANOVA)

The P value is 0.0232, considered significant. Variation among column medians is significantly greater than expected by chance.

The P value is approximate (from chi-square distribution) because exact calculations would have taken too long.

Kruskal-Wallis Statistic KW = 9.514

Dunn's Multiple Comparisons Test

| Comparison | Mean Rank Difference | P value |
|----------------------------|----------------------|-----------|
| S+NVP 6h vs. LPS+NVP 6h | 5.600 | ns P>0.05 |
| S+NVP 6h vs. S+NVP 24h | 9.800 | ns P>0.05 |
| LPS+NVP 6h vs. LPS+NVP 24h | -5.400 | ns P>0.05 |
| S+NVP 24h vs. LPS+NVP 24h | -9.600 | ns P>0.05 |

Appendix I-7: P-values of NVP plasma concentrations of groups G (S+NVP) and H (LPS+NVP) during the chronic phase

Kruskal-Wallis Test (Nonparametric ANOVA)

The P value is 0.0143, considered significant.
Variation among column medians is significantly greater than expected by chance.

The P value is approximate (from chi-square distribution) because at least one column has two or more identical values.

Kruskal-Wallis Statistic KW = 14.222 (corrected for ties)

Dunn's Multiple Comparisons Test

| Comparison | Mean Rank | |
|-----------------------------------|------------|-----------|
| | Difference | P value |
| S+NVP 7days vs. LPS+NVP 7days | 0.2500 | ns P>0.05 |
| S+NVP 7days vs. S+NVP 14days | 10.250 | ns P>0.05 |
| S+NVP 7days vs. S+NVP 21days | 14.083 | ns P>0.05 |
| LPS+NVP 7days vs. LPS+NVP 14days | 4.700 | ns P>0.05 |
| LPS+NVP 7days vs. LPS+NVP 21days | -2.000 | ns P>0.05 |
| S+NVP 14days vs. LPS+NVP 14days | -5.300 | ns P>0.05 |
| S+NVP 14days vs. S+NVP 21days | 3.833 | ns P>0.05 |
| LPS+NVP 14days vs. LPS+NVP 21days | -6.700 | ns P>0.05 |
| S+NVP 21days vs. LPS+NVP 21days | -15.833 | * P<0.05 |

APPENDIX J: PUBLICATIONS

Appendix J-1: Conference abstracts

Poster presentations

Z. Bekker, A. Walubo and J.B. du Plessis. **A Method for Determination of Nevirapine in a Small Plasma Volume by High Performance Liquid Chromatography.** Faculty Forum 2009, Faculty of Health Sciences of the University of the Free State, Bloemfontein, South Africa, 27 – 28 August 2009. Also at 5th Annual Conference of the South African Society for Basic and Clinical Pharmacology, Potchefstroom, South Africa, 23 – 26 September 2009.

Z. Bekker, A. Walubo and J.B. du Plessis. **The Effect of Acute and Chronic Administration of Nevirapine on Interleukin-2 in a Rat Model.** The 16th World Congress of Basic and Clinical Pharmacologists (WorldPharma 2010), Copenhagen, Denmark, 17 – 23 July 2010.

Oral presentation

Z. Bekker, A. Walubo and J.B. du Plessis. **The Effect of Acute and Chronic Administration of Nevirapine on Interleukin-2 in a Rat Model.** Faculty Forum 2010, Faculty of Health Sciences of the University of the Free State, Bloemfontein, South Africa, 26 – 27 August 2010.

A Method for Determination of Nevirapine in a Small Plasma Volume by High Performance Liquid Chromatography

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Aim:

Nevirapine (NVP) is an antiretroviral agent that is used for prophylaxis and treatment of HIV. Unfortunately, its use is associated with occurrence of hypersensitivity reactions and hepatotoxicity, the mechanism of which remains unclear. Furthermore, NVP is an enzyme inducer that is metabolised by cytochrome P450, and as such, it is liable to P450-drug interactions during combination therapy with other drugs, particularly the protease inhibitors, leading to toxic or sub therapeutic concentrations. Therefore, the use of NVP requires plasma concentration monitoring for which a method for analysis is required. Unfortunately, the existing methods could not be adopted owing to the solvents and instrumentation used, while the rapid methods posed a problem regarding the column-life. Therefore, a high performance liquid chromatography (HPLC) assay for determination of NVP concentrations in plasma was developed.

Methods:

Sample preparation: To 100 µl of plasma spiked with NVP, chlorzoxazone (IS) was added and proteins were precipitated with perchloric acid, then centrifuged, and the supernatant was purified by solid phase extraction on C18 cartridges with acetonitrile:water (80:20, v/v), and 40 µl of the eluent was injected into the HPLC.

HPLC conditions: The sample was analysed on HP 1100 series with an isocratic pump and a UV detector set at 210nm. The mobile phase was TEAP buffer and acetonitrile (60:40; v/v) at a flow rate of 1 ml/min. Separation of the compounds was performed on a C₁₈, 5 micron (150x4.6 mm) analytical column with a run time of 8 minutes.

Method Validation: Calibration curves (0 µg/ml – 10 µg/ml) were run over 5 days and the linear regression and correlation coefficient (r) were calculated using the GraphPad® statistical program. Accuracy was tested at 1, 5 and 10 µg/ml, while stability was tested at room temperature, 4°C and -20°C at 8 hours, 12 hours, 24 hours and 1 week.

Results:

Nevirapine eluted at 2.6 min. while the IS eluted at 5.2 min., and both peaks were sharp and symmetrical. The average 5 days calibration curve was linear ($y = 0.012x + 0.051$; $r = 0.9985$) with a CV% of $\pm 5.25\%$, while accuracy at 1, 5 and 10 µg/ml was 89.9%, 100.6% and 96.3%, respectively. Stability at -20°C was 91.7% and 86.1% at 24 hours and 1 week, respectively. The method was used to monitor NVP in rat plasma.

Conclusion:

An accurate and effective HPLC method for measurement of NVP in a small plasma volume (100 µl) was developed. This method will be useful for analysis of NVP in patients, particularly in newborns and in small research animals (mice and rats) where a small volume of blood is often available.

The Effect of acute and chronic administration of nevirapine on interleukin-2 in a rat model

Zanelle Bekker, A. Walubo and J.B. du Plessis

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South Africa.

Nevirapine (NVP) induced hepatotoxicity is regarded as partly due to hypersensitivity reactions, implying activation of the immune system. Therefore the aim of this study was to determine the effect of acute and chronic NVP administration on interleukin-2 (IL-2), a cytokine implicated in cell-mediated toxic-immune reactions, after subclinical immune stimulation with bacterial lipopolysaccharide (LPS). Male Sprague-Dawley rats were used and the study was approved by the animal ethics committee. During the acute phase, 2 groups of 10 rats received NVP (200mg/kg) after pre-treatment with either normal-saline (control) or LPS (test-group). Five animals were sacrificed at 6 and 24 hours after NVP administration. For the chronic phase, 2 groups of 15 rats each received NVP (200mg/kg) daily over 3 weeks. After every 7 days, 5 rats from each group were pre-treated with either normal-saline (control) or LPS (test-group) and sacrificed 24 hours later. Serum IL-2 was measured by ELISA. Although not statistically significant, during the acute phase, IL-2 concentration (pg/ml: mean±sd) was higher in the LPS-treated-group than in the control group (421.6±65.7 vs. 386.1±33.4 at 6 hrs; and 398.6±42 vs. 368.9±16.6 at 24 hrs), but during chronic administration, IL-2 concentration (pg/ml) was lower in the LPS-treated-group than in the control group at week 1 (298.8±10.8 vs. 359.7±62.9), week 2 (351.9±41.4 vs. 378.9±60.3) and week 3 (313.1±23.2 vs. 469.4±143.1). These results imply that NVP is a slow-onset IL-2 stimulant as was observed during chronic administration, and that this was augmented in the acute phase by LPS. Further studies are needed on the toxicologic significance of these observations.

THE EFFECT OF ACUTE AND CHRONIC ADMINISTRATION OF NEVIRAPINE ON INTERLEUKIN-2 (IL-2) IN A RAT MODEL

Z. Bekker, A. Walubo and J.B. du Plessis

Introduction and Aim

Nevirapine (NVP) induced hepatotoxicity is regarded as partly due to hypersensitivity reactions, implying activation of the immune system. Therefore the aim of this study was to determine the effect of acute and chronic NVP administration on interleukin-2 (IL-2), a cytokine implicated in cell-mediated toxic-immune reactions, after subclinical immune stimulation with bacterial lipopolysaccharide (LPS).

Materials and Methods

Male Sprague-Dawley rats were used and the study was approved by the Animal Ethics Committee. During the acute phase, 3 groups of 10 rats received NVP (200mg/kg), LPS and normal-saline (S) as follows: S+NVP, LPS+S and LPS+NVP. Five animals were sacrificed at 6 and 24 hours after dosing. For the chronic phase, 2 groups of 15 rats each received NVP (200mg/kg) daily over 21 days. After every 7 days, 5 rats from each group were treated with either normal-saline (S+NVP) or LPS (LPS+NVP) and sacrificed 24 hours later. Serum IL-2 was measured by ELISA.

Results

During the acute phase, IL-2 concentration (pg/ml) was highest in the NVP+LPS group compared to the S+NVP and LPS+S-groups (421.6 ± 65.7 vs. 386.1 ± 33.4 and 317.4 ± 64.6 at 6 hours; 398.6 ± 42.9 vs. 368.9 ± 16.6 and 517.9 ± 152.3 at 24 hours), but during chronic administration, IL-2 concentration (pg/ml) was higher in the S+NVP-group than in the LPS+NVP-group at day 7 (359.7 ± 62.9 vs. 298.8 ± 10.8), day 14 (378.9 ± 60.3 vs. 351.9 ± 41.4) and day 21 (469.4 ± 143.1 vs. 313.1 ± 23.2).

Conclusion

These results imply that NVP is a slow-onset IL-2 stimulant that was observed during chronic administration, and that this was augmented in the acute phase by LPS. Further studies are needed to determine on the toxicologic significance of this observation.

Appendix J-2: Manuscripts in preparation

1. **Bekker Z and Walubo A. Determination of nevirapine in a small plasma volume by high performance liquid chromatography.** Journal of Chromatography B. Submitted February 2010.
2. **Bekker Z and Walubo A. The role of the immune system in nevirapine induced hepatotoxicity.** British Journal of Pharmacology. Submitted February 2010.

SUMMARY

Key terms: nevirapine, hepatotoxicity, immune system, high performance liquid chromatography, lipopolysaccharide, cytokines.

Nevirapine is associated with hypersensitivity reactions such as skin rash and hepatotoxicity, hampering its use for HIV prophylaxis. The mechanism of nevirapine induced hepatotoxicity remains unknown and was postulated to be immune mediated. As recently reported, several drugs have shown to induce toxicity by immune activation. Therefore, the aim of this study was to determine the role of the immune system in nevirapine induced hepatotoxicity.

A high performance liquid chromatography method for the determination of nevirapine in rat plasma was developed. It involved protein precipitation with perchloric acid, followed by solid phase extraction on C₁₈ cartridges. The mobile phase was tetraethylammoniumphosphate (TEAP) buffer and acetonitrile (60:40, v/v) and was run over a Luna C₁₈ (4.60x150 mm) 5 μ analytical column at 1 ml/min. The eluent was detected by UV at 210 nm. Nevirapine and chlorzoxazone eluted at 2.6 and 5.2 minutes, respectively. The average 5 day calibration curve (0 – 10 μ g/ml) was linear with a regression equation of $y=0.012x+0.051$, and the correlation coefficient (r^2) was 0.9985. The method was used to measure nevirapine concentrations in rat plasma.

The animal experiment consisted of an acute and chronic phase. During the acute phase male Sprague-Dawley rats were divided into 3 groups of 10 rats each. Groups were dosed as follows: S+NVP, LPS+S and LPS+NVP. From each group, 5 rats were sacrificed at 6 and 24 hours after dosing. During the chronic phase, animals were divided into 2 groups of 15 rats each, to which nevirapine was administered daily. From each group, 5 rats were administered with LPS or saline, just before the daily nevirapine dose on days 7, 14 and 21. The animals were sacrificed 24 hours later. Blood was tested

for ALT, full blood count, IL-2, IFN- γ , TNF- α and nevirapine concentrations. Liver sections were sent for histopathology testing.

In the acute phase, the S+NVP group exhibited increased ALT at 6 and 24 hours. Liver injury was characterised by mild cloudy swelling (6 hours), and hepatocellular vacuolar degeneration (24 hours). Although LPS+S led to increased ALT at 6 hours, which normalised by 24 hours, abnormal liver histology was observed on both occasions as swollen cytoplasm and narrow sinusoids. The LPS+NVP group showed increased ALT at 6 hours, which returned to normal by 24 hours. Interestingly, liver histology was normal on both occasions, indicating that LPS+NVP prevented hepatotoxic effects of either drug. Whereas all three groups caused increased IL-2, it was higher in the LPS+NVP group, implying that LPS was responsible for the increase during the acute phase. Nevirapine concentrations at 6 hours were higher in the S+NVP group, but by 24 hours they were higher the LPS+NVP group. During the chronic phase, S+NVP caused liver injury on days 7 and 14 as demonstrated by increased ALT with centrilobular hepatocellular degeneration on day 7. However, by day 14 and 21 the ALT had normalised and liver histology was unaffected. Whereas the LPS+NVP group exhibited increased ALT on days 7 and 14, the corresponding liver histology was normal on all three occasions, illustrating further that LPS attenuates nevirapine induced hepatotoxicity. By day 21, S+NVP caused profound lymphocytosis, while LPS+NVP caused neutrophilia. S+NVP and LPS+NVP exhibited increased IL-2 and IFN- γ , but both cytokines were higher in the S+NVP group. TNF- α was elevated in both groups on day 7, and thereafter fell, remaining higher in the LPS+NVP group. Nevirapine concentrations were higher in the LPS+NVP group than in the S+NVP group.

In conclusion, it was demonstrated that nevirapine is a slow onset immune stimulant, which caused nevirapine induced hepatotoxicity, and LPS prevented the hepatotoxicity. These observations suggest that immune manipulation may help to prevent nevirapine induced hepatotoxicity.

OPSOMMING

Sleuteltermes: nevirapien, hepatotoksisiteit, immuunsisteem, hoëdrukvlloeistof-chromatografie, lipopoliesakkaried, sitokiene.

Nevirapien word geassosiëer met hipersensitiwiteitsreaksies soos veluitslae en hepatotoksisiteit wat die gebruik daarvan vir MIV profilakse belemmer. Die meganisme van nevirapien-geïnduseerde hepatotoksisiteit is onbekend en word gepostuleer om immuun-bemiddeld te wees. Soos onlangs aangemeld, veroorsaak verskeie middels hepatotoksisiteit deur immuun-aktivering. Die doel van hierdie studie was dus om die rol van die immuunsisteem in nevirapien-geïnduseerde hepatotoksisiteit te bepaal.

'n Hoëdrukvlloeistof-chromatografiemethode vir die bepaling van nevirapien in rotplasma is ontwikkel. Dit behels proteïen presipitasie met perchloraatsuur, gevolg deur vastefaseëkstraksie met C₁₈ kolom. Die mobiele fase het bestaan uit tetraetielammoniumfosfaat (TEAP) buffer en asetonitriël (60:40, v/v) en was gechromatografeer deur 'n Luna C₁₈ (4.60x150 mm) 5µ analitiese kolom teen 1 ml/min. Die eluant is bepaal deur UV teen 210 nm. Nevirapien en chlorzoksasoon het onderskeidelik teen 2.6 en 5.2 minute gëelueer. Die gemiddelde 5 dag kalibrasiekromme (0 – 10 µg/ml) was liniêr met 'n regressievergelyking van $y=0.012x+0.051$, en die korrelasie-koëffisiënt (r^2) was 0.9985. Die metode is suksesvol gebruik om nevirapienkonsentrasies in rotplasma te bepaal.

Die diereëksperiment het bestaan uit 'n akute- en chroniese fase. Gedurende die akute fase is manlike Sprague-Dawley rotte opgedeel in 3 groepe van 10 rotte elk. Die groepe is as volg behandel: saline+nevirapien, lipopoliesakkaried+saline en lipopoliesakkaried+nevirapien. Uit elke groep is 5 rotte geslag teen 6 en 24 uur na middeldoediening. Gedurende die chroniese fase is die diere opgedeel in 2 groepe van 15 rotte elk, waaraan nevirapien daaglik toegedien is. Uit elke groep is 5 rotte behandel met LPS

of saline, net voor die daaglikse nevirapiëndosis, op dae 7, 14 en 21. Die diere is 24 uur later geslag. Bloed is getoets vir ALT, volbloedtelling, IFN- γ , IL-2, TNF- α en nevirapienkonsentrasies. Lewermonsters is gestuur vir histopatologiese ondersoek.

In die akute fase het die S+NVP groep verhoogde ALT gedemonstreer teen 6 en 24 uur. Lewerskade was gekarakteriseer deur ligte swelling (6 uur) en hepatosellulêre vakuolêre degenerasie (24 uur). Alhoewel LPS+S gelei het tot verhoogde ALT teen 6 uur, wat herstel het teen 24 uur, was abnormale lewerhistologie waargeneem as geswolle sitoplasma en vernoude sinusoïedes tydens beide intervalle. Die LPS+NVP groep vertoon verhoogde ALT teen 6 uur, wat genormaliseer het teen 24 uur. Dit was merkwaardig dat die lewerhistologie normaal was tydens beide gevalle, wat aandui dat LPS+NVP die hepatotoksiese effekte van beide middels voorkom het. Al drie groepe het verhoogde IL-2 veroorsaak, met die hoogste vlakke in die LPS+NVP groep, wat aandui dat LPS verantwoordelik was vir die verhoging tydens die akute fase. Nevirapien konsentrasies was hoër in die S+NVP groep teen 6 uur, maar teen 24 uur was dit hoër in die LPS+NVP groep. Gedurende die chroniese fase het S+NVP lewerskade veroorsaak teen 7 en 14 dae, soos aangetoon deur verhoogde ALT met sentrilobulêre hepatosellulêre degenerasie teen dag 7. Teen dae 14 en 21 het die ALT genormaliseer en die lewerhistologie was normaal. Alhoewel die LPS+NVP groep verhoogde ALT getoon het teen dae 7 en 14, was die lewerhistologie normaal tydens al 3 intervalle, wat weereens aandui dat LPS nevirapiengeïnduseerde hepatotoksiteit geatenuer het. Teen dag 21 het S+NVP merkwaardige limfositose veroorsaak, terwyl LPS+NVP gelei het tot neutrofilie. Beide S+NVP en LPS+NVP het gelei tot verhoogde IL-2 en IFN- γ , maar beide sitokiene was hoër in die S+NVP groep. TNF- α was verhoog in beide groepe teen dag 7, waarna dit gedaal het, maar was steeds die hoogste in die LPS+NVP groep. Nevirapienkonsentrasies was hoër in die LPS+NVP groep as in die S+NVP groep.

Gevolgtik is daar gedemonstreer dat nevirapien 'n stadige imuunstimulant is en nevirapien-geïnduseerde hepatotoksisiteit veroorsaak het, en dat LPS nevirapien-geïnduseerde hepatotoksisiteit voorkom het. Die waarnemings stel voor dat immuunmanipulasie mag help om nevirapien-geïnduseerde hepatotoksisiteit te voorkom.