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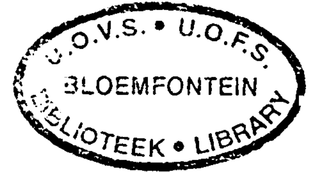

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THE ROLE OF CYP3A IN NEVIRAPINE INDUCED HEPATOTOXICITY

A DISSERTATION SUBMITTED FOR THE MASTER OF MEDICAL SCIENCE (M.MED.SC) DEGREE IN PHARMACOLOGY

IN ACCORDANCE WITH THE REQUIREMENTS OF THE FACULTY OF HEALTH SCIENCES, DEPARTMENT OF PHARMACOLOGY AT THE UNIVERSITY OF THE FREE STATE

By

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ABSTRACT

Nevirapine is a potent non-nucleoside reverse transcriptase inhibitor with favourable pharmacokinetics that are characterised by rapid absorption and distribution with a long elimination half-life. Nevirapine is effective against HIV-1 when used in combination with other anti-retroviral agents and as a monotherapy for the prevention of mother-to-child-transmission. Unfortunately, its adverse effects, mainly hypersensitivity skin reactions and hepatotoxicity, hamper the wide use of nevirapine. Since nevirapine-induced hepatotoxicity commonly occurs between 2 –12 weeks of treatment, and nevirapine is a known inducer of CYP3A isozyme, it was envisaged that the hepatotoxicity was due to activation of nevirapine to toxic metabolites by the induced enzyme. Therefore, the aim of this study was to determine the role of CYP3A in nevirapine-induced hepatotoxicity.

Fifteen male SD rats were pre-treated with either dexamethasone (50 mg/kg) or nevirapine (20 mg/kg) for 3 days. On the fourth day, the control group (n=5) was administered with the vehicle, while the 'nevirapine only' group (n = 5) was given an overdose of nevirapine (1340 mg/kg, orally) and the 'ketoconazole plus nevirapine' group (n = 5) was treated with a CYP3A inhibitor (ketoconazole) 1 hour before the overdose of nevirapine was given. The animals were sacrificed 24 hrs later and plasma was sent for liver function tests, the liver was excised for microsomal extraction and histopathology studies. Microsomal CYP3A activity was measured using the erythromycin demethylation test. Results of these animals were compared with results obtained in rats that were not pre-treated with an inducer before the toxic dose of nevirapine was administered.

Treatment with dexamethasone or nevirapine lead to increased CYP3A activity. CYP3A activity in the untreated, dexamethasone treated and nevirapine treated rats was 0.59 ± 0.48, 10.39 ± 3.59 and 7.28 ± 2.65 nmol/min/mg protein, respectively. In the dexamethasone pre-treated groups, the histopathological findings as well as the elevated liver enzymes of the 'nevirapine only' treated group (AST 239.25 ± 50.7 U/L and ALT ± 386.00 ± 154.3 U/L) were indicative of hepatotoxicity as opposed to the control group (AST 146.40 ± 10.4 U/L and ALT 149.60 ± 39.7 U/L). However, the

corresponding group pre-treated with nevirapine did not show significant elevations in liver enzymes (AST 150.20 ± 19.1 U/L and ALT 67.20 ± 8.6 U/L) but the histopathological findings exhibited hepatotoxicity that was similar to the dexamethasone group. Nevirapine-induced hepatotoxicity was not prevented in the groups treated with ketoconazole before the overdose of nevirapine was given. Interestingly, there was no hepatotoxicity when the overdose of nevirapine was administered to animals that were not pre-treated with nevirapine or dexamethasone.

In conclusion, nevirapine-induced hepatotoxicity was associated with enzyme induction by dexamethasone or nevirapine, and the use of ketoconazole did not prevent the hepatotoxicity. Therefore, CYP3A may not be involved in the pathogenesis of nevirapine-induced hepatotoxicity, suggesting that a different enzyme may be responsible. As the liver function tests did not correlate well with the histopathological findings in the nevirapine pre-treated groups, it was suggested that liver function tests alone might not be good markers for determining nevirapine-induced hepatotoxicity.

DECLARATION OF INDEPENDENT WORK

I, Shera Barr, 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 / faculty. I furthermore cede copyright of the dissertation in favour of the University of the Free State.

Barr
Signature

28 Feb 2004
Date

SUPERVISOR'S DECLARATION

I, Prof. A. Walubo, the supervisor of this dissertation entitled: The role of CYP3A in nevirapine induced hepatotoxicity, hereby certify that the work in this project was done by Shera Barr at the department of Pharmacology, University of the Free State.

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

Signature

Date

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TABLE OF CONTENTS

ABSTRACT i
DECLARATION OF INDEPENDENT WORK iii
SUPERVISOR'S DECLARATION iv
ACKNOWLEDGEMENTS v
ABBREVIATIONS xiii
LIST OF FIGURES xv
LIST OF TABLES xviii

GENERAL INTRODUCTION 1
LITERATURE REVIEW 3

PART I: HIV, AIDS AND TREATMENT 3
2.1 Human Immunodeficiency Virus (HIV) 3
2.1.1 HIV Structure and components 3
2.1.2 HIV target for infection 4
2.1.3 HIV life cycle 5
2.1.3.1 HIV entry and fusion 5
2.1.3.2 Synthesis of viral DNA 5
2.1.3.3 Integration 6
2.1.3.4 Synthesis of viral mRNA and proteins 6
2.1.4 HIV transmission 8
2.1.5 HIV diagnosis 8
2.1.6 Stages of HIV disease 8
2.2 Acquired Immune Deficiency Syndrome 9
2.2.1 Anti-retroviral therapy for HIV 10
2.2.1.1 Nucleoside Analogue Reverse Transcriptase Inhibitors 10
2.2.1.2 Non-nucleoside Reverse Transcriptase Inhibitors (NNRTI's) 10
2.2.1.3 Protease Inhibitors 10
2.2.1.4 Fusion inhibitors 11
2.3 Nevirapine 13
2.3.1 Indication 13

2.3.2 Chemistry and classification 13
2.3.3 Antiviral activity 13
2.3.4 Pharmacokinetics 14
2.3.5 Advantages 15
2.3.6 Side effects 16
2.3.7 Drug interactions 16
2.3.8 Resistance 16

PART II: DRUG METABOLISM 17
2.4 Microsomal mixed function oxidase system 19
2.5 Cytochrome P450 enzymes 19
2.5.1 General Characteristics 20
2.5.2 Location 20
2.5.3 Nomenclature 20
2.5.4 Function 21
2.6 CYP450 Enzyme induction 21
2.6.1 Methods for determining CYP450 induction (Okey, 1990) 22
2.6.1.1 Pharmacologic effects 22
2.6.1.2 Measuring P450 concentration 22
2.6.1.3 An increase in the catalytic activity 22
2.6.1.4 Increased levels of P450, measured by immuno-quantitation 23
2.6.1.5 Increased levels of mRNA and transcription, Northern blot analysis 23
2.6.2 Consequences of CYP450 induction 23
2.7 Enzyme inhibition 23
2.8 Factors involved in the expression of CYP proteins and in drug biotransformation 25
2.8.1 Genetic differences 25
2.8.2 Disease 25
2.8.3 Age 25
2.8.4 Gender 25
2.9 CYP 3A Family 26
2.10 Methods for determining CYP3A activity 28
2.10.1 Probe drugs 28
2.10.2 Erythromycin Demethylation 28

PARTIII: IMMUNOCHEMICAL TECHNIQUES USED FOR PROTEIN DETECTION29

2.11 Immunoassays 29

2.11.1 Radioimmunoassay (RIA)...29

2.11.2 Enzyme-linked immunosorbent assay (ELISA).......................................30

2.12 Electrophoresis and immunoblotting 31

2.12.1 Electrophoresis 31

2.12.2 Electrotransfer 32

2.12.3 Immunoblotting / immunodetection 35

PART IV: DRUG INDUCED HEPATOTOXICITY 38

2.13 Drug induced hepatotoxicity...38

2.13.1 Classification of Hepatotoxins..39

PART V: DIAGNOSIS OF LIVER DISEASE 42

2.14 Biochemical tests / Liver function tests (LFTs)..42

2.14.1 Important liver enzymes commonly measured...42

2.14.2 Serum Transaminases..43

2.14.2.1 Alanine aminotransferase (ALT) 43

2.14.2.2 Aspartate aminotransferase (AST) 43

2.14.2.3 Alkaline phosphatase (ALP) 44

2.15 Pathologic diagnosis of Hepatic disease 44

OBSERVATIONS FROM REVIEW, HYPOTHESIS AND OBJECTIVES 45

3.1 Observations from review 45

3.2 Hypothesis..45

3.3 Objectives...45

3.3.1 Determining CYP3A activity in rat microsomes..45

3.3.2 Establishing the optimum time for CYP3A enzyme induction in rats using nevirapine and dexamethasone as inducers...45

3.3.3 Determining the role of CYP3A inhibitors in preventing nevirapine-induced hepatotoxicity. 45

THE MEASUREMENT OF RAT MICROSOMAL CYP3A ACTIVITY IN VITRO........46

4.1 Introduction...46

4.2 Reagents **46**
4.2.1 Special buffers and prepared reagents 47
4.3 Apparatus **47**
4.4 Animals **47**
4.5 Part I: Animal experiments **47**
4.5.1 Methods 47
4.5.1.1 Surgical procedure and blood collection 47
4.5.1.2 Microsomal preparation 48
4.5.1.3 Determination of microsomal protein concentration 48
4.5.2 Results 49
4.6 PART II: Unexpected low CYP3A activity **50**
4.6.1 Methods 50
4.6.1.1 Reaction conditions for CYP3A assay 50
4.6.1.2 Sample preparation 51
4.6.1.3 Troubleshooting 51
4.6.2.2 Comment 53
4.6.3 Results 53
4.7 Discussion **56**

STANDARDISATION OF REACTION CONDITIONS FOR THE ASSAY OF RAT MICROSOMAL CYP3A ACTIVITY **57**
5.1 Introduction **57**
5.2 Reagents **57**
5.3 Apparatus **57**
5.4 Methods **57**
5.4.1 Assay for CYP3A activity *in vitro* 57
5.4.2 Calibration for the determination of formaldehyde content in microsomal solutions. 58
5.4.3 Methods for standardisation of reaction conditions 58
5.4.3.1 Microsomal protein content 58
5.4.3.2 Time of incubation 59
5.4.3.3 Substrate concentration 59
5.5 Results **59**
5.5.1 Formaldehyde Calibration results 59

5.5.2 Results for the standardisation of reaction conditions 61
5.5.2.1 Microsomal protein content 61
5.5.2.2 Time of incubation 63
5.5.2.3 Substrate concentration 65
5.6 Discussion 67

DETERMINING THE OPTIMUM TIME OF INDUCTION OF RAT CYP3A 68
6.1 Introduction 68
6.2 Reagents 68
6.3 Method 68
6.3.1 Treatment with dexamethasone 68
6.3.2 Treatment with nevirapine 69
6.3.3 Analysis of results 69
6.4 Results 70
6.4.1 Treatment with dexamethasone 70
6.4.2 Treatment with nevirapine 70
6.4.3 Comparison of dexamethasone and nevirapine treated rats 71
6.5 Discussion 75

CYP3A QUANTITATION BY SDS-PAGE AND WESTERN BLOTTING 76
7.1 Introduction 76
7.2 Reagents 76
7.3 Apparatus 77
7.4 Methods 77
7.4.1 Preparation of buffers 77
7.4.2 Sample preparation 78
7.4.3 SDS PAGE and CYP3A detection by Western blotting 78
7.4.3.1 Gel preparation 78
7.4.3.2 Electrophoresis 79
7.4.3.3 Electrotransfer 79
7.4.3.4 Blotting 79
7.4.3.5 ECL detection 80
7.4.4 Optimisation of experimental conditions 80
7.4.5 The final adjustments to the procedure for CYP3A detection 84

7.5 Effect of dexamethasone and nevirapine on CYP3A 85
7.6 Results 85
7.6.1 Troubleshooting 86
7.7 Discussion 89

THE ROLE OF CYP3A INHIBITORS IN THE PREVENTION OF NEVIRAPINE INDUCED HEPATOXICITY 90
8.1 Introduction 90
8.2 Reagents and apparatus 90
8.3 Methods 91
8.3.1 Animal experiment 91
8.3.2 Statistical analysis 91
8.3.3 PART I: TEST FOR NEVIRAPINE INDUCED HEPATOTOXICITY IN NORMAL RATS 91
8.3.3.1 Experiment 91
8.3.3.2 Results 91
8.3.3.3 Comment 94
8.3.4 PART II: PRE-TREATMENT WITH NEVIRAPINE 95
8.3.4.1 Experiment 95
8.3.4.2 Results 95
8.3.4.3 Histopathological findings of rats pre-treated with nevirapine. 99
8.3.4.4 Comment 101
8.3.5 PART III: PRE-TREATMENT WITH DEXAMETHASONE 102
8.3.5.1 Experiment 102
8.3.5.2 Results 103
8.3.5.3 Histological findings of rats pre-treated with dexamethasone. 107
8.3.5.4 Comment 110

DISCUSSION 111
CONCLUSIONS 114
FUTURE STUDIES 115
REFERENCES 116
APPENDICES 131
Appendix A 132

Appendix B 138
Appendix C 142
Appendix D 143
Appendix E 144
Appendix F: Conference abstract 146

OPSOMMING 148
SUMMARY 150

ABBREVIATIONS

Ab	antibody
Abs	absorbance
AIDS	Acquired Immune Deficiency Syndrome
ALT	alanine aminotransferase
ALP	alkaline phosphatase
AMPS	ammonium persulfate
AST	aspartate aminotransferase
BSA	bovine serum albumin
CA	capsid
Cal	calibration
CV	coefficient of variation
CYP450	cytochrome P450
DDL	diacetyl dihydrolutidine
DMSO	dimethylsulphoxide
DNA	deoxyribonucleic acid
EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme-linked immunosorbent assay
EM	erythromycin
GGT	gammaglutamyl transpeptidase
HIV	Human Immunodeficiency Virus
HPLC	high performance liquid chromatography
HRP	horseradish peroxidase
IgM	immunoglobulin
MA	matrix
MAb	monoclonal antibody
mRNA	messenger ribonucleic acid
NADP	nicotineamide adenine dinucleotide phosphate
NC	nucleocapsid
NNRTI	non-nucleoside reverse transcriptase inhibitor
NVP	nevirapine
PAGE	polyacrylamide gel electrophoresis

PBS	phosphor buffered saline
PVDF	polyvinylidene difluoride
RIA	radioimmunoassay
RNA	ribonucleic acid
SD	standard of deviation
SDS	sodium dodecyl sulphate
TEMED	N, N, N', N' - tetramethylenediamine
Tris	tris(hydroxymethylaminomethane)
UV	ultra-violet

LIST OF FIGURES

Figure 2.1 Structure of the HIV-1 virion 4
Figure 2.2 Interaction of HIV gp120 with the CD4 receptor 6
Figure 2.3 Life cycle of HIV 7
Figure 2.4 Schematic course of HIV infection 9
Figure 2.5 Structure of nevirapine 13
Figure 2.6 *In vitro* biotransformation of nevirapine 15
Figure 2.7 Anionic properties of SDS that uniformly provides proteins with a negative charge 32
Figure 2.8 The assembly of the gel sandwich 34
Figure 2.9 The production of chemiluminescence in the presence of horseradish peroxidase 35
Figure 2.10 A schematic representation of the detection of proteins, using enzyme labelled antibodies 36
Figure 2.11 Schematic representation of the SDS-PAGE and western blotting technique 37

Figure 4.1 Calibration curve of BSA standards, $y = -0.009x + 1.0054$, $r^2 = 0.9896$ 50
Figure 4.2 The average activity of microsomal CYP3A in untreated and dexamethasone treated rats 55

Figure 5.1 The average calibrations of formaldehyde, the product of erythromycin demethylation 60
Figure 5.2 A plot of CYP3A activity at varying protein concentrations 62
Figure 5.3 A plot of the amount of the metabolite produced at varying times of incubation 64
Figure 5.4 A plot of CYP3A activity at varying erythromycin (substrate) concentrations 66

Figure 6.1 CYP3A activity after treatment with dexamethasone, 30 and 50 mg/kg/day 72

Figure 6.2 CYP3A activity after treatment with nevirapine, 20 and 40 mg/kg/day ..74

Figure 7.1 Western blot of CYP3A indicating signals of the different amounts of microsomal protein loaded (0.5, 1, 2.5, 5, 10 and 15 μg), after incubation with primary antibody dilution of 1:2500 82
Figure 7.2 Western blot of CYP3A indicating signals of the different amounts of microsomal protein loaded (0.1, 0.25, 0.5, 1 and 2.5 μg) after incubation with primary antibody dilution of 1:2500 82
Figure 7.3 Western blot of CYP3A supposed to indicate signals of the different amounts of microsomal protein loaded (0.1, 0.25, 0.5, 1 and 2.5 μg) after incubation with primary antibody dilution of 1:5000......................83
Figure 7.4 Western blot of CYP3A supposed to indicate signals of the different amounts of microsomal protein loaded (0.75, 1, 1.5 and 2. μg). Secondary antibody dilution was 1:2000..83
Figure 7.5 Western blot of CYP3A indicating signals of the different amounts of microsomal protein loaded (0.75, 1, 1.5 and 2. μg). Electro-transfer was for 2 hours at 100 V..84
Figure 7.6 A blot of CYP3A in microsomes from untreated rats 86
Figure 7.7 A blot of CYP3A in microsomes from rats treated with nevirapine for 3 days (A) and 5 days (B)..87
Figure 7.8 A blot of CYP3A in microsomes from rats treated with dexamethasone for 3 days (A) and 5 days (B)...88

Figure 8.1 Liver function tests for rats in the control group (Group A), the group treated with 'nevirapine only' (Group B) and the group treated with the 'inhibitor and nevirapine' (Group C)...93
Figure 8.2 Liver function tests of rats in the control group (Group D), the 'nevirapine only' treated group (Group E) and the 'ketoconazole plus nevirapine' group (Group F)..98
Figure 8.3 Histological characteristics of a rat liver in the control group, showing mild cellular hypertrophy. Liver enzymes for this specific animal were, in U/L; ALP 190, AST 155 and ALT 79...100

Figure 8.4 Histological characteristics of a rat liver in the 'nevirapine only' treated group, hepatocellular hypertrophy and apoptosis was detected. Liver enzymes of this specific animal were, in U/L; ALP 151, AST 175 and ALT 56 100

Figure 8.5 Liver function tests of rats in the control group (Group H), 'nevirapine only' treated group (Group I) and 'ketoconazole plus nevirapine' treated group (Group L) 106

Figure 8.6 Histology of a rat liver in the control group, showing mild hepatocellular degeneration with some vacuolation in the cytoplasm. No vacuolar hepatopathy or severe vacuolization of the hepatocytes. Liver enzymes for this specific animal were, in U/L; ALP 121, AST 153 and ALT 211 108

Figure 8.7 Histology of a rat liver in the 'nevirapine only' treated group, showing hepatocellular hypertrophy. Liver enzymes for this specific animal were, in U/L; ALP 144, AST 190 and ALT 112 108

Figure 8.8 Histological characteristics of a liver treated with the inhibitor, showing similar findings as the vehicle group. No vacuolar hepatopathy, hepatocellular hypertrophy or apoptosis was detected. Liver enzymes for this specific animal were, in U/L; ALP128, AST 135 and ALT 158 . 109

Figure 8.9 Histological characteristics of the liver treated with the inhibitor and nevirapine, hepatocellular hypertrophy and apoptosis was detected. Liver enzymes for this specific animal were, in U/L; ALP166, AST 620 and ALT 416 109

LIST OF TABLES

Table 2.1 Properties of some anti-HIV drugs 12
Table 2.2 Reactions involved in Phase I and Phase II metabolic pathway............18
Table 2.3 Location of certain CYP enzymes in the body......................................20
Table 2.4 Factors affecting the activity of certain CYP450 isoforms......................26
Table 2.5 Substrates, inhibitors and inducers of CYP3A......................................27
Table 2.6 Comparison of the properties of ELISA and RIA 31
Table 2.7 Properties of membranes used for western blotting techniques............33
Table 2.8 Various blocking agents used to block non-specific sites on membranes. 35
Table 2.9 A few examples of liver lesions due to drugs and toxins 40
Table 2.10 Serum markers for liver disease...43

Table 4.1 Absorbance values of the corresponding standards.............................49
Table 4.2 The activity of microsomal CYP3A (nmol/min/mg protein) in untreated and dexamethasone pre-treated rats 54

Table 5.1 Formaldehyde calibrations performed on different days........................59
Table 5.2 CYP3A activity at various protein concentrations.................................61
Table 5.3 Amount of metabolite (formaldehyde) produced at various incubation times...63
Table 5.4 CYP3A activity at varying substrate (erythromycin) concentrations 65

Table 6.1 CYP3A activity (nmol/min/mg protein) after 3 and 5 days of treatment with dexamethasone, 30 mg/kg/day 71
Table 6.2 CYP3A activity (nmol/min/mg protein) after 3 and 5 days of treatment with dexamethasone, 50 mg/kg/day 71
Table 6.3 CYP3A activity (nmol/min/mg protein) after 3 and 5 days of nevirapine treatment, 20 mg/kg/day...73
Table 6.4 CYP3A activity (nmol/min/mg protein) after 3 and 5 days of nevirapine treatment, 40 mg/kg/day...73

Table 8.1	Liver function tests for rats in the control group (Group A) and the group treated with 'nevirapine only' (Group B).	92
Table 8.2	Liver function tests of rats treated with 'nevirapine only' and those treated with the 'ketoconazole plus nevirapine'	92
Table 8.3	Liver function tests of rats in the control group (Group D) and those in the 'ketoconazole only' treated group (Group E)	96
Table 8.4	Liver function tests of rats in the control group (Group D) and those in the 'nevirapine only' treated group (Group F)	96
Table 8.5	Liver function tests of rats in the 'nevirapine only' treated group (Group E) and the 'ketoconazole plus nevirapine' treated group (Group F)	97
Table 8.6	Liver function tests of the control group (Group H) and the nevirapine only group (Group I)	103
Table 8.7	Liver function tests of rats in the control group (Group H) and those treated with ketoconazole (10 mg/kg, Group J)	104
Table 8.8	Liver function tests of rats in the control group (Group H) and those treated with ketoconazole (20 mg/kg, Group K).	104
Table 8.9	Liver function tests of rats treated with 'nevirapine only' (Group I) and those treated with ketoconazole (10 mg/kg) and nevirapine (Group L)	105

CHAPTER 1

GENERAL INTRODUCTION

The human immunodeficiency virus (HIV) is one of the leading causes of deaths among young adults today. The virus attacks the immune system and the body becomes vulnerable to many opportunistic diseases, resulting in the development of an acquired immune deficiency syndrome (AIDS). The treatment of HIV remains a mystery and as of date there is no cure. Therefore, treatment is aimed at improving and prolonging quality of life. Currently, there are many available drugs that have been approved for the use in HIV treatment. They are classified according to their mechanism of action; these include the nucleoside reverse transcriptase inhibitors, protease inhibitors and non-nucleoside reverse transcriptase inhibitors.

Nevirapine is a potent non-nucleoside reverse transcriptase inhibitor that was recently added to the list of current treatments. This drug is specific and effective against HIV-1 when used in a combination therapy as well as monotherapy for the prevention of mother to child transmission (Cheeseman *et al.*, 1993; Mirochnick *et al.*, 2000). Nevirapine has advantages over the other available anti-retroviral agents, as it is cost effective, active in its native state and it is administered to individuals with ease. Unfortunately, its adverse effects have hampered its wide use, as long-term use of nevirapine in adults has resulted in significant side effects including life-threatening hepatotoxicity and severe skin reactions.

Since understanding the mechanism of nevirapine-induced toxicity is a pre-requisite for developing effective preventive strategies, it was suggested that further research regarding this field needed to be undertaken.

To understand the mechanism of nevirapine induced hepatotoxicity, the pharmacokinetic and pharmacodynamic profile of the drug had to be viewed in a manner to make possible association with its toxicokinetic profile. It was interesting to note that the hepatotoxic events of nevirapine commonly occur between 2-12 weeks of therapy, implying that the drug may not be directly toxic to the liver. The drug also has an exceptionally long elimination half-life of 30 - 45 hrs (Mirochnick *et al.*, 2000;

Martínez *et al.* 2001), also suggesting that only when the drug is metabolised, is when toxicity may be experienced. The aforementioned statements indicate that hepatotoxicity was possibly due to the metabolic products of nevirapine.

Taking a closer look at the metabolic pathway of nevirapine which has been well documented (Riska *et al.*, 1999a), it was noted that nevirapine is extensively biotransformed into various metabolites by CYP450 enzymes, with isozyme CYP3A being the major enzyme involved. Interestingly, nevirapine also induces its own metabolism via CYP3A and CYP2B6. Considering that nevirapine induces CYP3A from 2-4 weeks of therapy and that the onset of nevirapine hepatotoxicity commonly occurs between 2 – 12 weeks of therapy, it was postulated that the CYP3A may play a role in nevirapine-induced hepatotoxicity.

Since CYP450 isozymes have been implicated in the bioactivation of drugs to toxic metabolites, e.g. the bioactivation of paracetamol (Park *et al.*, 1995), and the knowledge of this has provided preventative strategies against this bioactivation, it was suggested that nevirapine-induced hepatotoxicity might be due to the bioactivation of the parent drug to a toxic metabolite. Because CYP3A is the major isozyme involved in nevirapine metabolism, it was decided to investigate the role of this CYP450 isozyme in nevirapine-induced hepatotoxicity. It was hoped that determining the mechanism of nevirapine-induced hepatotoxicity would provide a means for the prevention thereof.

CHAPTER 2

LITERATURE REVIEW

PART I: HIV, AIDS AND TREATMENT

2.1 Human Immunodeficiency Virus (HIV)

HIV is a known cause of the Acquired Immune Deficiency Syndrome (AIDS) that is characterised by the depletion of CD4-positive lymphocytes. HIV is a member of the retrovirus family *Retroviridae* and belongs to the genus *Lentiviridae*, also known as a lentivirus (Meissner and Coffin, 1989; Haseltine, 1991). Most lentiviruses or "slow" growing viruses are characterised by a long interval between the start of infection and the onset of symptoms. As retroviruses only contain RNA, they are unable to replicate outside a living host cell. HIV therefore attacks the immune system and leaves the body vulnerable to many ordinary illnesses that can cause fatal outcomes (Meissner and Coffin, 1989).

AIDS is caused by two distinct but related human strains namely HIV-1 and HIV-2. HIV-1 is the most common and infectious variant that has led to a worldwide HIV epidemic. The HIV-2 variant is less common and less virulent but similar clinical findings to HIV-1 are eventually produced (Koenig and Fauci, 1990).

2.1.1 HIV Structure and components

HIV is made up of a lipid bilayer consisting of surface (gp120) and transmembrane (gp 41) glycoproteins. This lipid bilayer encloses the interior, which is composed of a matrix (MA), capsid (CA) and the nucleocapsid (NC) proteins. At least nine proteins are encoded by the genes of HIV, which are found in the central part of the proviral DNA (Frankel and Young, 1998; Haase 1990). These proteins are categorised as major structural proteins, regulatory proteins and accessory proteins (Hope and Trono, 1999; Frankel and Young, 1998; Young, 1999). Figure 2.1 shows the schematic representation of the structure of HIV with its essential components.

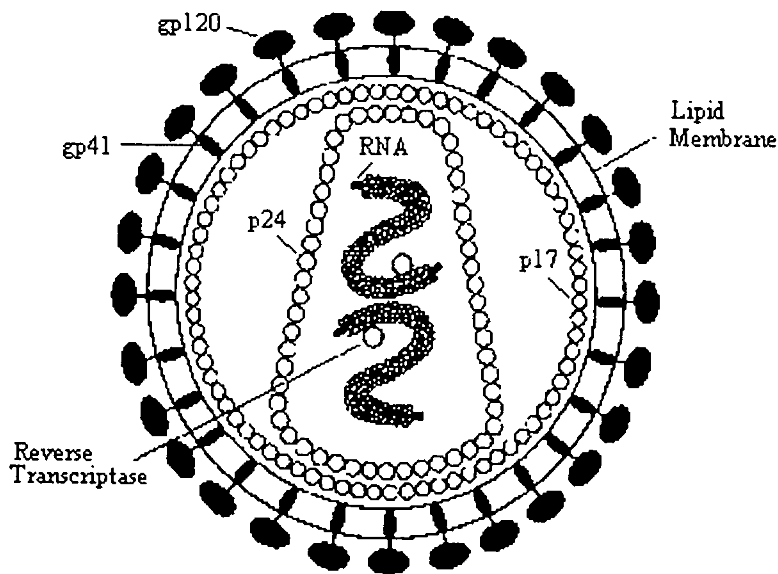


Figure 2.1 Structure of the HIV-1 virion (Available from http://hivpositive.com/f-HIV-you).

The structural genes, gag, env and pol are responsible for the formation of the basic HIV components. The **env** gene is synthesised as a precursor glycoprotein (gp160), which is cleaved into the outer glycoproteins, gp120 and gp41 (Kowalski *et al.*, 1987). The glycoprotein gp120 is responsible for tropism to CD4+ receptors, and transmembrane gp41 catalyses the fusion of HIV to the target cells (Caffrey, 2001). The **gag** gene directs the formation of the matrix proteins, p17, the capsid p24 and the nucleocapsid p7. The **pol** gene instructs the synthesis of important enzymes including reverse transcriptase, integrase and protease.

HIV encodes 6 additional proteins, the regulatory proteins, **Tat** and **Rev**, and the accessory proteins, **Nef**, **Vpr**, **Vpu** and **Vif** (Peterlin and Trono, 2003; Hope and Trono, 1999). These proteins are responsible for the functioning and regulation of viral expression (Frankel and Young, 1998; Hope and Trono, 1999).

2.1.2 HIV target for infection

HIV-1 and HIV-2 primarily infect and destroy cells via the CD4 receptor molecule, also known as the binding site for HIV-1. This receptor has been well characterised in human and simian immunodeficiency viruses (Young, 1999) as it contains four immunoglobulin-like domains (Landau and Warton, 1988). The CD4 protein is

present on the surface of T-lymphocytes in high concentration and on monocytes, macrophages and dendritic cells in low concentration (Haseltine, 1991; Rosenberg and Fauci, 1991).

2.1.3 HIV life cycle

2.1.3.1 HIV entry and fusion

The entry of HIV occurs through fusion of the host and virus cell membranes, which is mediated by the viral envelope glycoproteins (Kwong *et al.*, 1998; Rizzuto *et al.*, 1998), but a co-receptor known as chemokine is needed for HIV fusion to the membrane (Fig. 2.2). Chemokines are cell surface fusion-mediated molecules that recruit leukocytes from circulation to the sites of infection (Horuk, 1999; Dalgleish *et al.*, 1984). According to differences in their structure and function, chemokine receptors fall into two categories, including CC and CXC. HIV binding is initially mediated by the conformational change of the gp120 subunit, but these changes are not sufficient for the actual fusion process. The chemokine produces a conformational change in the gp41 subunit, allowing the actual fusion of HIV to take place. Different chemokine co-receptors are responsible for the selective infection by different strains of HIV (Horuk, 1999). The T-tropic strains, for example, interact with the CXCR4 chemokine co-receptor to infect lymphocytes, whereas the M-tropic strains interact with the CCR5 chemokine co-receptor to infect macrophages (Berger and Murphy, 1998; Asthana *et al.*, 2001). Once the virus has fused with the host's membrane, viral components are then emptied into the cell's cytoplasm, after which the subsequent steps of the HIV life cycle take place.

2.1.3.2 Synthesis of viral DNA

HIV is a retrovirus containing only RNA and therefore HIV is unable to replicate outside of living host cells. When the virus enters the cell, the viral genome is released into the host cell, where it uses its reverse transcriptase enzyme to transcribe its RNA to DNA (Varmus, 1988; Frankel and Young, 1998, Peterlin and Trono, 2003). Figure 2.3 shows a schematic representation of the life cycle of HIV.

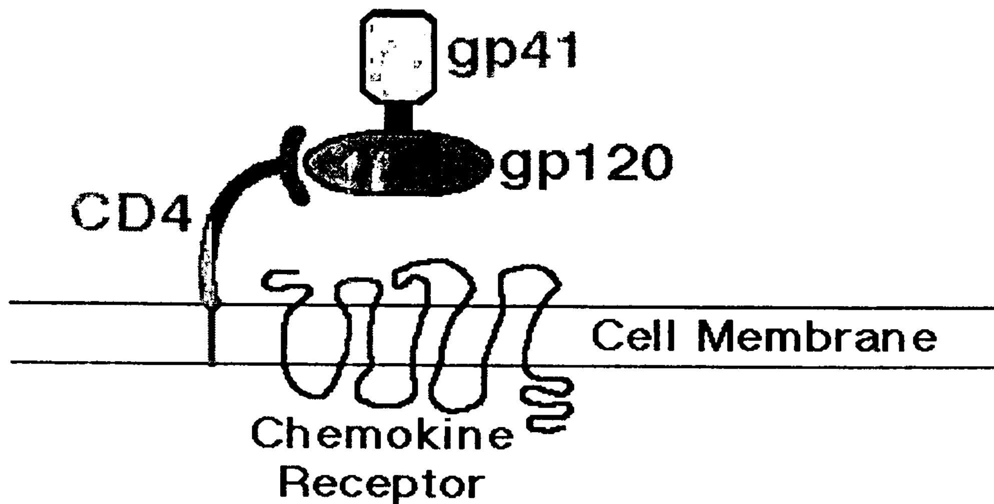


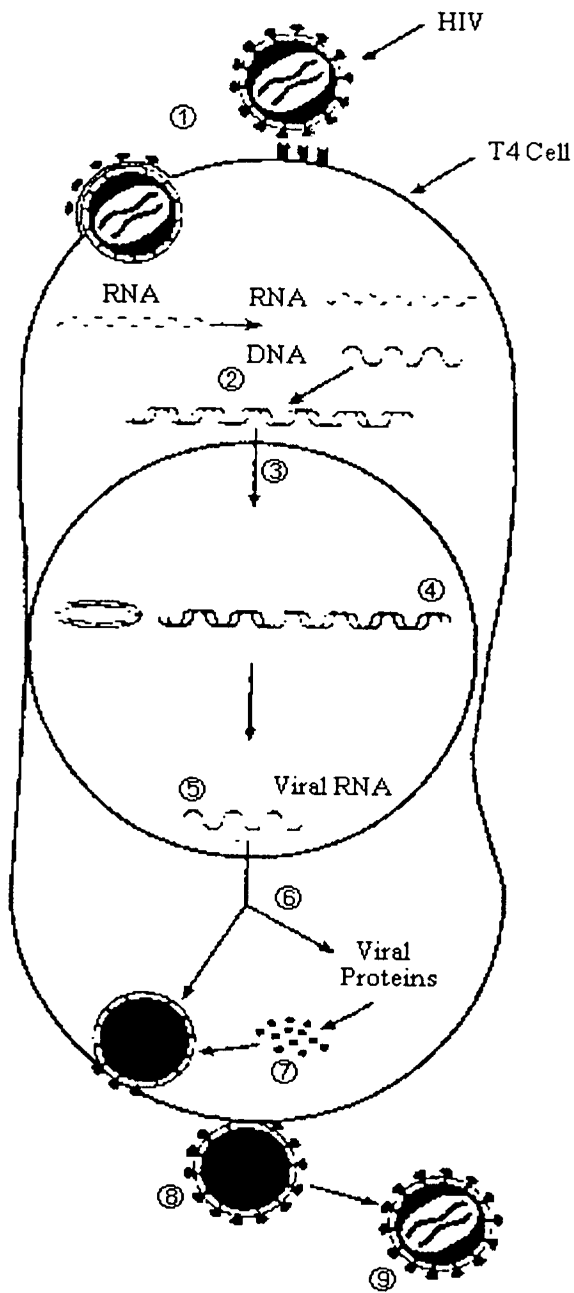
Figure 2.2 Interaction of HIV gp120 with the CD4 receptor (Klatt, 1994)

2.1.3.3 Integration

The virus has its own enzyme, integrase, which facilitates the incorporation of its DNA into the host DNA. The integrated DNA is referred to as a proviral DNA. The proviral DNA may remain dormant for many years or it may become activated (Frankel and Young, 1998; Hope and Trono, 1999).

2.1.3.4 Synthesis of viral mRNA and proteins

The viral DNA is transcribed to yield copies of viral messenger RNA by the host. The messenger RNA is then translated into viral proteins (Haseltine, 1991). The mRNA is then escorted from the nucleus to the cytoplasm where it is translated into structural proteins, such as Gag, Pol and Env. Several of the regulatory proteins assist in the production of these viral proteins, while the maturation of the virion is mediated by viral proteases (Frankel and Young, 1998). Once the virions have been produced they aggregate near the cell surface where they bud off from the host cell.



Steps in Viral replication

1. Attachment
2. Reverse Transcription and DNA Synthesis
3. Transport to nucleus
4. Integration
5. Viral Transcription
6. Viral Protein Synthesis
7. Assembly of Virus
8. Release of Virus
9. Maturation

Figure 2.3. Life cycle of HIV (Available from http://hivpositive.com/f-HIV-you).

2.1.4 HIV transmission

HIV is a sexually transmitted virus that has also been isolated in saliva, tears, nervous system tissue, blood, semen, vaginal fluid and breast milk. However, successful transmission is attained when the virus is in high concentrations as in infected blood, semen or vaginal secretions (Starr and Taggart, 1995).

2.1.5 HIV diagnosis

The diagnosis of HIV is based on identifying the HIV-1 antibody or structural proteins found in the blood. The enzyme linked immmunosorbent assay (ELISA) and western blotting techniques are commonly applied for detecting HIV antibodies. HIV RNA tests as well the p24 antigen assay are also commonly used in clinical settings to diagnose primary HIV infection before antibodies to HIV are detectable (Schwartz *et al.*, 1988; Burke *et al.*, 1988; Constantine, 1999).

2.1.6 Stages of HIV disease

HIV infection occurs in different stages, acute, latent and symptomatic stage. Figure 2.4 depicts the stages of HIV infection and the development of AIDS. Once HIV infects an individual, it affects numerous CD4+ cells and replicates rapidly. During this acute phase, the amount of CD4+ cells declines rapidly and after 2-4 weeks of exposure to the virus the infected individual presents with flu-like symptoms relating to this acute stage. The immune system of the individual responds to the infection by producing antibodies that dramatically reduce the levels of HIV. This results in the latent stage, where the viral load becomes stable and the patient progresses to a long latent phase lasting for several years. The patient remains asymptomatic but the virus is never truly latent; active HIV is found in lymph nodes, lymphoid organs and macrophages at all stages of infection (Weiss, 1993). As the infection progresses and the viral load increases to very high levels, the CD4+ count decreases and symptoms of fever, malaise and weight loss occur, thereby increasing the risk of opportunistic infections, which are in most cases fatal. Age, genetic differences, the HIV strain and virulence of the virus may influence the rate and progression of the disease.

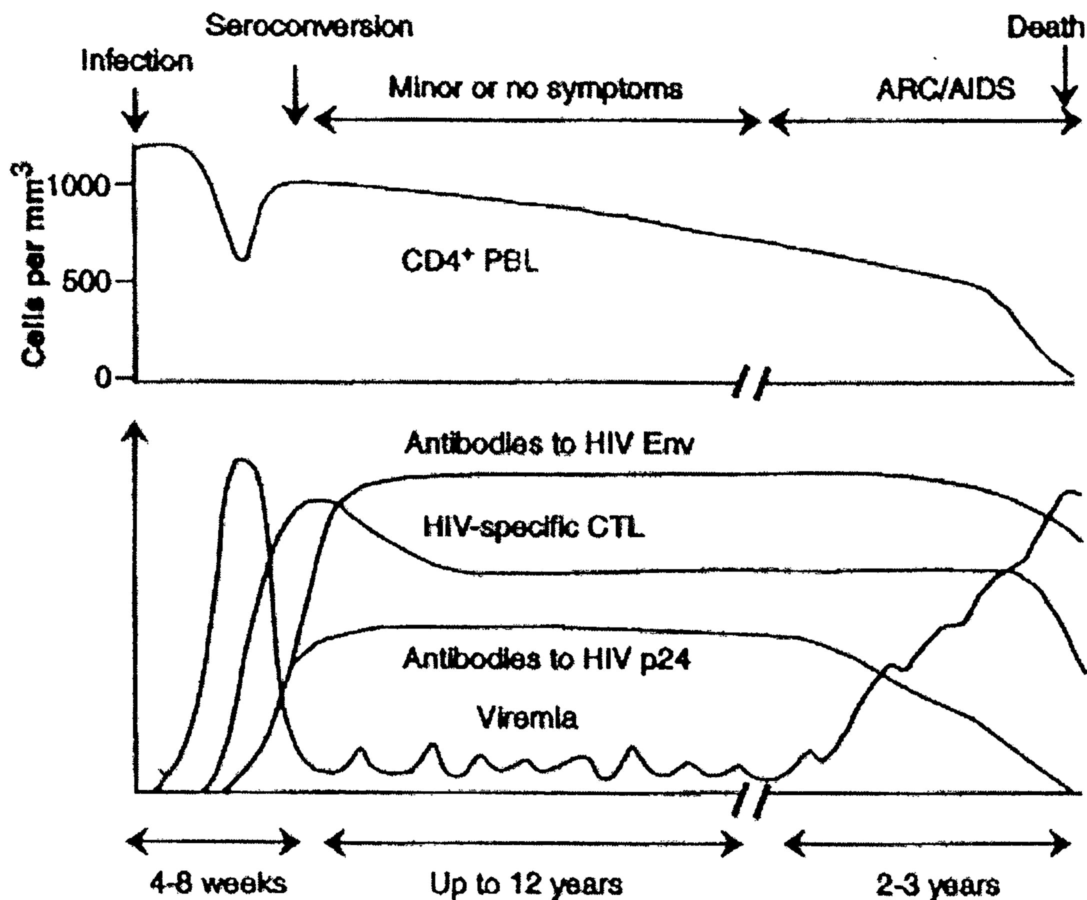


Figure 2.4 Schematic course of HIV infection (Weiss, 1993).

2.2 Acquired Immune Deficiency Syndrome

AIDS caused by HIV is the final and most serious stage of HIV infection, that is characterised by the depletion of CD4+ lymphocyte cells in the blood (Weiss, 1993). For several years the infected persons have no symptoms but they are still capable of transmitting the disease to others. Acute HIV infection progresses over time from the early symptomatic infection to asymptomatic infection and then later to AIDS (Klatt, 1994, Cohen, 1999).

The symptoms of AIDS are primarily due to infections that don't normally develop in healthy individuals; these infections are termed "opportunistic infections". With suppression of the host's immune response by the AIDS virus, opportunistic organisms are free to cause disease; the most common symptoms are fevers, diarrhoea, sweats, chills, weakness and weight loss (Meissner and Coffin, 1989).

The severity of damage to the immune system is measured by an absolute CD4 lymphocyte count. The CD4 lymphocyte is an essential cell in the blood stream that assists in the prevention of several cancers and infections. When an infected individual has a CD4 count of less than 200cells/μl they are said to have AIDS, the most fatal stage of HIV infection (Klatt, 1994).

In addition to the CD4 lymphocyte count, there are other tests that are useful in managing the HIV disease and progress, e.g. T lymphocyte count, chest x-rays and Pap smears.

2.2.1 Anti-retroviral therapy for HIV

2.2.1.1 Nucleoside Analogue Reverse Transcriptase Inhibitors:

Didanosine, lamivudine, stavudine, zalcitabine and zidovudine are examples of the nucleoside analogues available for the treatment of HIV infection. Nucleoside analogues mimic nucleotides and when incorporated into viral DNA it causes chain termination (Hirsch and D'Aquilla, 1993). These nucleoside analogues are sometimes classified by the nucleotides they mimic and they work better when used in combination (Ritter *et al.*, 1999, Deeks and Volberding, 1999; Fischl, 2001). Table 2.1 shows the anti-retroviral agents used currently in HIV treatment.

2.2.1.2 Non-nucleoside Reverse Transcriptase Inhibitors (NNRTI's):

Delavirdine, efavirenz and nevirapine are examples of NNRTI's. These NNRTI's interfere with the action of the HIV reverse transcriptase enzyme by binding to the enzyme and preventing it from functioning (Ritter *et al.*, 1999; Deeks and Volberding, 1999; von Moltke *et al.*, 2001).

2.2.1.3 Protease Inhibitors:

These drugs interfere with the protease enzyme responsible for the newly formed HIV polyproteins. When this enzyme's function is inhibited, the HIV does not produce viral surface proteins because they don't contain the viral components, therefore they cannot infect other cells. Indinavir, saquinavir, nelfinavir and amprenavir are examples of the available protease inhibitors (Ritter *et al.*, 1999, Deeks and Volberding, 1999).

2.2.1.4 Fusion inhibitors

The fusion inhibitors are a new class of anti-retrovirals that have activity against HIV that is resistant to other drugs. Fusion inhibitors block the entry of HIV into the host cell (Boyle, 2001b). These drugs mimic the gp41 and inhibit fusion of HIV with the host cell, resulting in reduced replication rate of the virus (Hossein, 2001; Nutley and Durham, 2001; Scottsdale, 2001). Fusion inhibitors include, T-20 and T-1249, which have been undergoing clinical trials. T-20 (enfuvirtide, Fuzeon) was recently approved by the European Commission as well as the FDA (Nutley and Dunham, 2003).

Table 2.1 Properties of some anti-HIV drugs (Ritter *et al.*, 1999).

Drug	Side effects	Pharmacokinetics
Nucleoside analogues:		
Didanosine	GIT upsets, hyperuricaemia, peripheral neuropathy, nausea and vomiting	Hepatic metabolism and renal excretion 50%. Absorption affected by pH.
Zalcitabine	Neuropathy, pancreatitis, stomatitis and rashes	Well absorbed 85% bioavailability.
Stavudine	Peripheral neuropathy	Well absorbed 86% bioavailability.
Lamivudine	Headache, fatigue, skin rashes and abdominal pains.	t½ of 3-6 hrs. Well absorbed.
Zidovudine	Haematological toxicity: granulocytopenia and anaemia.	>90% absorbed from gut. Major metabolite (80%) is glucuronide.
NNRTI's:		
Delavirdine	Headaches and rashes.	t½ of 4-6 hrs. Hepatic metabolism by CYP450.
Nevirapine	Hepatitis and skin rashes.	t½ of 25-30 hrs. Metabolised in liver by CYP3A4. Autoinduction
Protease Inhibitors:		
Indinavir	Hepatic dysfunction and hyperbilirubinemia.	t½ of 2 hrs. Hepatic metabolism.
Saquinavir	GIT upsets, hyperglycaemia and hepatitis.	Poorly absorbed and hepatic metabolism.
Nelfinavir	GIT hyperglycaemia	98% protein bound and hepatic metabolism.
Amprenavir	Skin rashes and GIT upsets.	t½ of 7-10 hrs. Metabolised in liver by CYP3A4

2.3 Nevirapine

2.3.1 Indication

Nevirapine (VIRAMUNE®) is a potent non-nucleoside reverse transcriptase inhibitor (NNRTI) specifically used for treatment of HIV-1 infected individuals where it is used in a combination with other anti-retroviral agents (Cheeseman *et al.,* 1993). Nevirapine is also used as a monotherapy for the prevention of mother-to-child HIV-1 transmission (Mirochnick *et al.,* 2000).

2.3.2 Chemistry and classification

Nevirapine is a benzodiazepine derivative (Fig. 2.5), a member of the dipyridodiazepinone class of compounds with a molecular weight of 266.3 (Mirochnick *et al.*, 2000). It is a weak base with a pKa of 2.8 and is highly lipophilic (Cheeseman *et al.*, 1993). Nevirapine is an off-white powder that is currently available as a 200 mg tablet as well as a 10 mg/ml suspension that is recommended for children.

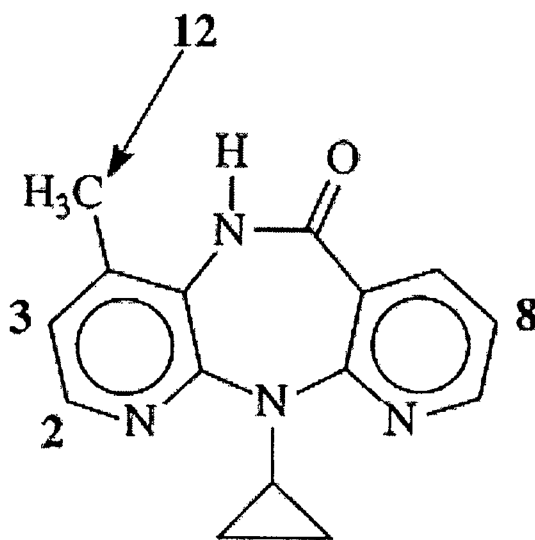


Figure 2.5 Structure of Nevirapine (Erickson *et al.*, 1999).

2.3.3 Antiviral activity

Nevirapine is specific for HIV-1, it inhibits the reverse transcriptase enzymes by binding directly to the tyrosine residues (positions 181 and 188), resulting into a reduced rate of HIV-1 synthesis (Cheeseman *et al.,* 1993 and Havlir *et al.*, 1995).

The major advantage of the non-nucleoside reverse transcriptase inhibitor drugs is their lack of cross-resistance with nucleoside reverse transcriptase inhibitors.

2.3.4 Pharmacokinetics

Nevirapine has been characterised with favourable pharmacokinetics in patients and healthy volunteers (Sabo *et al*, 2002). Nevirapine is well absorbed orally (>90 %), readily crosses the placenta, is found in breast milk and has a wide tissue distribution including the central nervous system (Mirochnick *et al*., 2000). From a pharmacokinetic perspective, age, race and gender are not major factors of nevirapine dose requirements.

Nevirapine is dependent upon metabolism for its elimination and it has been shown by *in vivo* and *in vitro* studies that it is extensively biotransformed to several hydroxylated metabolites via CYP450 enzymes with a small fraction of the dose excreted unchanged in the urine (Riska *et al*., 1999a, Riska *et al*., 1999b). *In vitro* studies have shown that CYP3A4 is primarily responsible for the metabolism of nevirapine, and CYP2B6, CYP2D6 and CYP2C9 to a lesser extent (Erickson *et al*, 1999). Figure 2.6 shows the different isoforms involved in nevirapine metabolism.

The half-life of nevirapine is approximately 45 hours, but with multiple dosing, autoinduction of the CYP450 enzymes CYP3A and CYP2B6 occurs, resulting in a reduced half-life of 25-30 hours due to increased rate of metabolism (Riska *et al*., 1999a; Piscitelli and Gallicano, 2001).

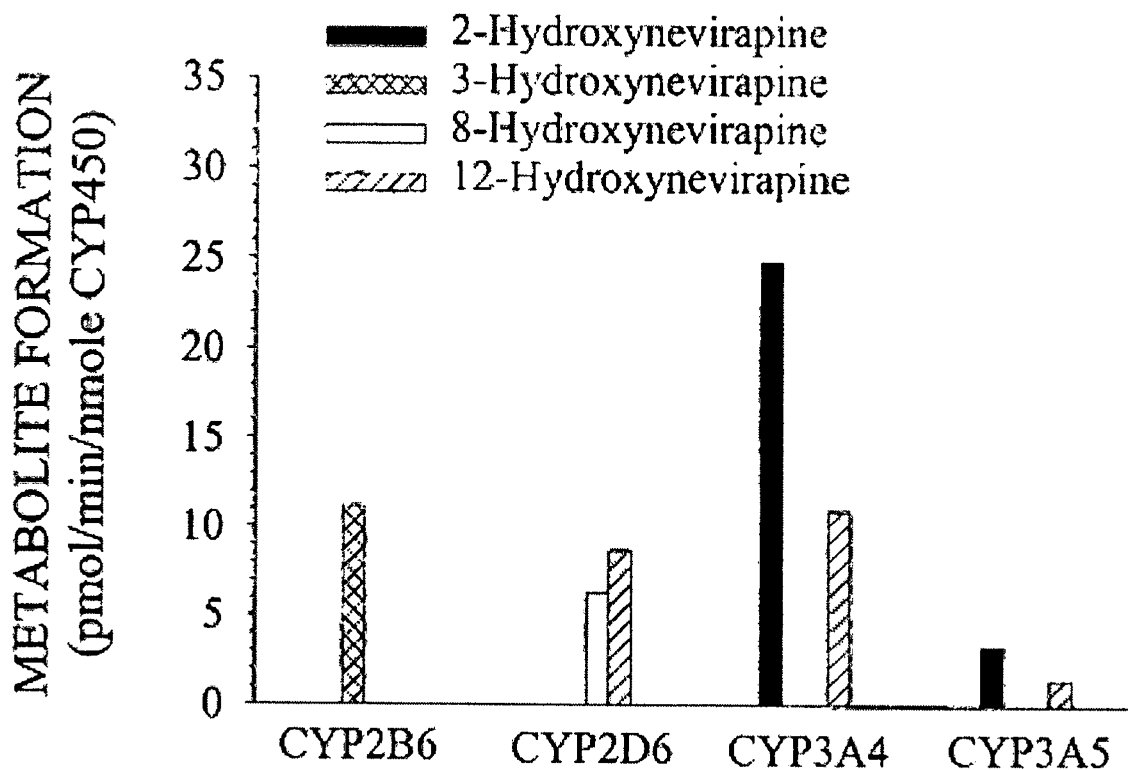


Figure 2.6 *In vitro* biotransformation of nevirapine (Erickson *et al.*, 1999)

2.3.5 Advantages

Nevirapine has advantages over the other currently approved anti-retroviral agents; the half-life is significantly longer, allowing less frequent administration and patient compliance. Another advantage of nevirapine is that it is active in its native state, inhibiting HIV-1 reverse transcriptase without requiring intracellular metabolism whereas Nucleoside Reverse Transcriptase Inhibitors (zidovudine) require intracellular phosphorylation before HIV replication is inhibited (Mirochnick *et al.*, 2000). The low cost of nevirapine (R ± R 250 / 240 ml Viramune®) and its simplicity of administration is advantageous over other expensive prevention treatments (Saloojee, 2002).

2.3.6 Side effects

Nevirapine treatment has raised controversial issues due to several cases of severe adverse effects that have been reported (Boyle, 2001a; Reisler *et al.*, 2001; Martinez *et al.*, 2001). Life threatening and some cases of fatal hepatotoxicity including fulminant and cholestatic hepatitis, hepatic necrosis and hepatic failure have been reported in patients treated with nevirapine (VIRUMINE®). Serious hepatic events occur most frequently during the first 12 weeks of therapy but cases have also been reported after 12 weeks. Serious hepatotoxicity has also been reported in HIV uninfected individuals receiving multiple doses of nevirapine in the setting of post-exposure prophylaxis, an unapproved use. Nevirapine is also responsible for another major side effect namely rashes, severe life-threatening skin reactions have been reported. Cases of Stevens-Johnson syndrome, toxic epidermal necrolysis and hypersensitivity reactions characterised by rash have also been reported (Martínez *et al.*, 2001; Mirochnick *et al.*, 2000)

2.3.7 Drug interactions

As nevirapine is a known inducer of CYP450 enzymes (Riska *et al.*, 1999a), the potential of drug interactions with certain agents exists. Nevirapine is an inducer of CYP3A4 and CYP2B6 (Sabo *et al.*, 2002; Cheeseman *et al.*, 1993; Riska *et al.*, 1999a) resulting in the reduction of plasma concentrations of concomitantly administered agents metabolised by the same enzymes. Since nevirapine induces CYP3A, it is known to reduce the efficacy of drugs that are metabolised by the same isoform, e.g. oral contraceptives as well as protease inhibitors (Mycek *et al.*, 1992; Mirochnick *et al.*, 2000; Ritter *et al.*, 1999; Piscitelli and Gallicano, 2001; Murphy *et al.*, 1999)

2.3.8 Resistance

Resistance to nevirapine develops rapidly when used as a monotherapy, due to mutations in the reverse transcriptase gene codon (Deeks and Volberding, 1999). The approach of HIV treatment has therefore been focussed on combining the different classes of anti-retroviral agents, an attempt to prevent viral replication from most angles (Fischl, 1994).

PART II: DRUG METABOLISM

Drug metabolism is an essential process that rids the body of foreign compounds. Understanding the biotransformation of a drug would provide significant information about the enzymes responsible for its metabolism and the respective metabolites.

The metabolism of drugs is dependent on the polarity of the molecules. Usually, polar molecules are generally poorly absorbed; however, once they are absorbed the kidneys readily excrete them. Non-polar molecules on the other hand penetrate membranes of the gut and skin, and are poorly eliminated by the kidneys because of reabsorption and protein binding. The non-polar nature of molecules needs to be decreased so that accumulation of these compounds is prevented in the body. The body is equipped with a number of metabolic pathways that assist in increasing the polarity of molecules for their elimination (Vessey, 1990).

Nearly every tissue has the ability to metabolise drugs, however, high concentrations of drug metabolising enzymes are present in the liver, making it one of the most essential organs involved in drug metabolism. The underlying principle of drug metabolism is to convert a drug into a more water-soluble compound and then to excrete it (Tredger and Stoll, 2002).

Drug metabolism may be Phase I or Phase II metabolism. Phase I metabolism increases the water solubility of the parent drug by introducing small polar groups (Tredger and Stoll, 2002; Vessey, 1990). These resulting metabolites are either inactive or modified. When these metabolites are sufficiently polar, they are easily excreted. However some metabolites may need to undergo further metabolism to become highly polar (Correia, 1995). Phase II metabolism introduces large polar molecules that are responsible for the formation of conjugates. Table 2.2 represents the reactions involved in these two categories.

Certain drugs can affect drug metabolising enzymes thereby leading to change in drug kinetics and response. The two major processes involved include enzyme induction or enzyme inhibition. Enzyme induction most often results in an increased

metabolic rate whereas enzyme inhibition, on the contrary, is characterised by a reduction in metabolism and usually occurs due to drugs competing for the same enzymatic site (Tredger and Stoll, 2002).

Table 2.2 Reactions involved in Phase I and Phase II metabolic pathways. (Tredger and Stoll, 2002).

	Metabolic reactions	Enzymes involved
Phase I reactions:		
Oxidation	Introduces hydroxyl, epoxide and ketone groups Converts alcohols to aldehydes and acids	Alcohol and aldehyde dehydrogenases Cytochrome P450
Reduction	Introduces hydrogen into ketones and nitro groups	Nitro- and azo-reductases
Hydrolysis	Breaks down esters to alcohols and acids	Esterases
Phase II reactions:		
Acetylation	Adds acetate to polar sites	Acetyltranferases
Glucuronidation	Adds sugars to polar sites	Glucuronyl transferases
Sulphation	Adds inorganic sulphate to polar sites	Sulphotransferases
Amino acid conjugation	Adds amino acid to polar sites	Glutathione transferases
Methylation	Adds methyl groups to polar sites	Methyltransferases

2.4 Microsomal mixed function oxidase system

Most drug-metabolising enzymes are located in the lipophilic membranes of the endoplasmic reticulum of the liver cells. These membranes, together with their enzymes, are usually isolated by means of tissue homogenisation and density gradient ultracentrifugation, and then referred to as microsomes (Correia, 1995).

Microsomes contain an essential class of enzymes referred to as mixed function oxidases (now CYP450) that require NADPH (reducing agent) as well as oxygen to function as catalysts of biochemical reactions. The initial description of the hepatic microsomal system responsible for the metabolism of xenobiotics was by Mueller and Miller (1953), who indicated that the microsomal fraction of the liver was responsible for the N-demethylation of aminoazo dyes and that a reductase was also present and shown to be responsible for the cleavage of the azo linkage (Mannering, 1968).

The advantage of using microsomes to determine the biotransformation of drugs is that they retain their morphologic and functional properties of the membrane (Correia, 1995). In the liver, the cytochrome P450 enzymes are the most abundant mixed function oxidases and therefore they are responsible for Phase I metabolism.

2.5 Cytochrome P450 enzymes

Cytochrome P450 enzymes are a system of mixed function oxidases responsible for the metabolism of a broad array of endogenous and exogenous compounds (Guengerich, 1994). This enzyme system has three major functions, first, they are essential drug metabolising enzymes that are responsible for the catalysis of Phase I metabolic reactions (Gonzalez and Nebert, 1990), facilitating the elimination and detoxification of metabolised products. Second, they have a role in the bioactivation of many xenobiotics and third, this system metabolises endogenous compounds, e.g. steroids, prostaglandins and fatty acids (Ryan and Levin, 1990; Guengerich, 1994).

2.5.1 General Characteristics

CYP450 are hydrophobic, intrinsic membrane proteins that are closely associated with intracellular membranes (Gonzalez, 1990). These enzymes are hemoproteins made up of between 400 and 500 amino acids and contain a single heme prosthetic group. They use the reduced form of NADPH and oxygen to oxidise substrates (Gonzalez, 1990).

2.5.2 Location

Cytochrome P450 enzymes are found in many tissues with the highest concentration located in the liver cells. Within the hepatocytes they are located in the membranes of the smooth endoplasmic reticulum (Goshman *et al.*, 1999 ; Omiecinski *et al.*, 1998). Table 2.3 represents the organ locations of some of the major cytochrome P450 enzymes present in humans.

Table 2.3 Location of certain CYP enzymes in the body.

Enzyme	Location
CYP1A1	Liver
CYP2D6	Liver and Brain
CYP3A4	Liver and Small Intestine
CYP3A5	Liver, Kidney and Leukocytes
CYP3A7	Placenta and Foetal Liver

2.5.3 Nomenclature

The name cytochrome P450 is derived from its heme composition and unusual spectrum. In the reduced state these enzymes are characterised by an absorption maximum of 450 nm, hence P450 (Omura and Sato, 1964).

The naming of the individual cytochrome P450 genes have been categorised into families and subfamilies. The root symbol "CYP" is followed by an Arabic symbol depicting the family of the isozyme e.g. CYP3. A letter denoting the subfamily

followed by another Arabic numeral indicates the specific individual isoform e.g. CYP3A4 (Badyal and Dadhich, 2001).

Isoenzymes of the same subfamily have >40 % homology in their amino acid sequence and those of the same subfamily have > 55 % homology (Nebert and McKinnon, 1994).

2.5.4 Function

The cytochrome P450 enzymes are responsible for the catalysis of phase 1 reactions, thereby assisting in the metabolism of significant drugs. The goal of the CYP450 mediated reactions is to make a drug more water-soluble so that the kidneys can excrete it. The drug or compound is inactivated during this process but sometimes the drug is converted into a more active metabolite e.g. losartan is activated by CYP2C9 and CYP3A while acetaminophen is converted to a hepatotoxic metabolite by CYP2E1 and CYP1A2 (Guengerich, 1994).

As cited earlier, besides the metabolism of drugs, CYP450 enzymes have many other functions, including steroid anabolism, metabolism of environmental toxins, dietary components and endogenous substances (Guengerich and Johnson, 1997).

2.6 CYP450 Enzyme induction

The induction of P450 by drugs was initially discovered through the reduction of a drug's pharmacological effects (Conney, 1967). CYP450 induction is defined as the increase in the activity and the amount of the drug metabolising enzyme, mainly from the CYP families 1-4 (Berthou, 2001). Induction of CYP enzymes can occur due to long-term pollutant exposure as well as the exposure to some drugs. Transcriptional activation is the most common mechanism of enzyme induction, leading to an increased expression of CYP proteins.

Some drugs are capable of inducing their own metabolism, this is referred to as autoinduction. Nevirapine is known to induce its own metabolism via CYP3A4 and CYP2B6 (Riska *et al.*, 1999a; Murphy *et al.*, 1999). Autoinduction results in reduced

drug intensity and duration, which can be problematic if the drug is metabolised to a toxic or active metabolite, as these toxic effects will be enhanced.

When CYP450 induction enhances detoxification, it would serve as a protective mechanism against toxicity of xenobiotics. However, when CYP450's metabolise drugs into toxic intermediates, induction may result in further toxicity. Induction of Phase I enzymes, in most cases results in insufficient detoxification by Phase II enzymes, toxicity therefore being dependent on the balance between the Phase I and Phase II enzymes (Berthou, 2001).

2.6.1 Methods for determining CYP450 induction (Okey, 1990)

Various methods are available for determining the induction of CYP450 enzymes. The ability to measure a specific isoform is clinically important, as it provides information about predictable and possible drug interactions that may result due to this induction.

2.6.1.1 Pharmacologic effects

A reduction in a drug's pharmacological response was initially used to explain enzyme induction in humans (Conney, 1967). Today, enzyme induction is known to increase the metabolism of a drug and decrease its pharmacological response, leading to a need for adjustment of dosages.

2.6.1.2 Measuring P450 concentration

The P450 spectrum provides a measure of total P450 content in tissue. The actual effects of induction were observed before the naming and discovery of P450 enzymes. This pigment was initially described by Klingenberg (1958) and Garfinkel (1958) and then named as P450 by Omura and Sato (1964) because of it having a unique peak in its reduced form at 450 nm. Enzyme induction increases the P450 concentration, which can be measured spectrally; the elevation in concentration is proportional to the increase in enzyme activity.

2.6.1.3 An increase in the catalytic activity

The increased P450 catalytic activity can be measured *in vitro* in microsomes from animals treated with specific inducers. The activity of an individual CYP isoform can

be measured using specific substrates (probes) for the respective enzyme (Berthou, 2001). Examples of probes include debrisoquine for the measurement of CYP2D6 activity, chlorzoxazone to measure CYP2E1 (Lucas *et al.*, 1999) and midazolam for determining CYP3A activity.

2.6.1.4 Increased levels of P450, measured by immuno-quantitation

Sensitive methods for the identification and quantitation of specific P450 isoforms have been developed of which immunochemical techniques are the most often used. Immunoblotting (Western blot) techniques have been used successfully to identify CYP450 and to determine the influence of inducers on the microsomal levels of the specific isozymes.

2.6.1.5 Increased levels of mRNA and transcription, Northern blot analysis.

The major mechanism of enzyme induction is the increase in transcription of the P450 gene, therefore, resulting in increased mRNA levels that can be analysed using the Northern blot technique.

2.6.2 Consequences of CYP450 induction

The consequences of P450 induction are primarily an altered pharmacological response to the drug. Induction leads to increased metabolism, which then leads to reduced efficacy of the therapeutic agent. In this case, dosages usually have to be adjusted to attain the desired therapeutic effect (Okey, 1990). Another consequence of P450 induction, is the possibility of toxicity as CYP450 enzymes can convert unreactive parent compounds into reactive intermediates that may be toxic or carcinogenic. Enzyme induction will therefore enhance this bioactivation, resulting in further toxicity or carcinogenicity.

2.7 Enzyme inhibition

Inhibition of CYP450 results in a decrease of the metabolism of one xenobiotic by another xenobiotic simultaneously present in the active site of the enzyme. Competitive inhibition is the most common mechanism of inhibition and occurs due to the competition between two drugs for the specific enzyme's binding site (Murray and Reidy, 1990; Tredger and Stoll, 2002). Drugs can however bind irreversibly

(mechanism based) or reversibly to an enzyme's binding site, preventing the binding of other substrates to that specific site.

Reversible inhibition can be of a competitive or non-competitive nature, depending on the binding capacity of the substrate and inhibitor as well as the concentration of the inhibitor (Badyal and Dadhich, 2001).

Irreversible (mechanism based) inhibition occurs when drugs are metabolised by the CYP450 enzymes to active metabolites that bind to the enzyme and result in irreversible loss of function. This is often known as suicide inhibition and depends on the total amount of inhibitor that is exposed to the CYP450 isozyme (Badyal and Dadhich, 2001). This type of loss in the enzyme's activity can only be restored by the synthesis of new enzymes, which may require several days (Tredger and Stoll, 2002).

The process of inhibition usually begins with the first dose of the inhibitor and the time needed for the offset of inhibition is dependent on the half-life of both drugs (Dossing *et al.*, 1983).

2.8 Factors involved in the expression of CYP450 proteins and in drug biotransformation

2.8.1 Genetic differences

The term polymorphism is used to describe the genetic trait responsible for the inter-individual and ethnic differences observed in the metabolic rate of certain drugs, very common for the CYP450 family of enzymes. CYP 2D6 is an example of an isozyme that has extensively been studied in this field and several populations have been characterised into poor and extensive metabolisers (Tredger and Stoll, 2002; Badyal and Dadhich, 2001; Goshman *et al.*, 1999).

2.8.2 Disease

An impaired liver has an effect on CYP450 enzymes, often leading to a decrease in the biotransformation of drugs. Expression of CYP1A2, CYP2E1 and CYP3A was shown to be reduced in cirrhotic patients (Badyal and Dadhich, 2001).

2.8.3 Age

The activity of the CYP450 enzymes is known to decrease with increasing age in both male and females. Specifically, the metabolism of drugs such as diazepam, lidocaine and theophylline is decreased in the elderly. Infants on the other hand have low enzyme activity at birth; these enzymes become mature at a young adult age and start to decrease at an old age (Badyal and Dadhich, 2001). On the contrary, Kinirons and Crome (1997) described that there is no decline in CYP450 quantity or activity with age and suggested that other factors other than CYP450 activity are responsible for the decrease in a drug's clearance with age, e.g. decreased renal function or other age-related disease.

2.8.4 Gender

Gender based differences in CYP450 expression have been identified in humans. It is known that women have a higher expression of CYP3A4 when compared to men, and this may predispose them to developing drug interactions (Badyal and Dadhich, 2001). Table 2.4 shows some factors effecting the expression of specific CYP450 activities and expression.

Table 2.4 Factors affecting the activity of certain CYP450 isoforms

Factors	CYP effected	Effect
Environmental factors:		
smoking	CYP1A2	Induce
Alcohol	CYP2E1	Induce
Pregnancy	CYP2D6	Induce

2.9 CYP 3A Family

The CYP3A family are the most abundant cytochrome P450 enzymes found in the liver. They account for about 30 - 60 % of the proteins in the liver and are also present in high levels in the small intestinal epithelium, therefore contributing to presystemic / first-pass metabolism (Goshman *et al.*, 1999; Badyal and Dadhich, 2001).

Approximately 40 – 50 % of clinically significant drugs are metabolised by CYP3A, with CYP3A4 being the major subfamily involved in this metabolism. The CYP3A family is composed of four major enzymes, CYP3A3, CYP3A4, CYP3A5 and CYP3A7, with CYP3A4 being the most predominant (Cupp and Tracy, 1998). These enzymes are closely related and are therefore often referred to collectively as CYP3A, the subfamily name. CYP3A is not only responsible for the metabolism of drugs but it is also significantly involved in the metabolism of endogenous steroids. As this subfamily represents the majority of CYP450 isoforms in the liver and because it metabolises a significant percentage of drugs, the possibility of drug interactions is quite high. Table 2.5 represents the relevant substrates, inhibitors and inducers of CYP3A. In contrast to CYP2D6, CYP3A is not polymorphic but shows great interindividual variation whereby some individuals have up to 60 % of their total P450 as CYP3A while others contain less than 10 % (Goshman *et al*, 1999; Shimada *et al.*, 1994).

Table 2.5 Substrates, inhibitors and inducers of CYP3A (Piscitelli and Gallicano, 2001; Michalets, 1998)

Substrates	Inhibitors	Inducers
Antiarrhythmics:	***Azole antifungals:***	Carbamazepine
Amiodarone	Itraconazole	Phenobarbitone
Lidocaine	Ketoconazole	Phenytoin
Propafenone	Fluconazole	Rifampicin
	Macrolide Antimicrobials:	Rifabutin
Benzodiazepines:	Erythromycin	Dexamethasone
Alprazolam	Clarithromycin	Nevirapine
Diazepam	***SSRIs:***	Troglitazone
Triazolam	Fluoxetine	
Calcium Channel blockers:	Paroxetine	
Diltiazem	***Calcium Channel blockers:***	
Nifedipine	Verapamil	
Verapamil	Diltiazem	
Antidepressants:	Nifedipine	
Amitriptyline	Protease inhibitors	
Clomipramine	Grapefruit Juice	
Imipramine	Cimetidine	
Antihistamines:	Propofol	
Terfenadine	***Antidepressants:***	
Astemizole	Nefazodone	
Miscellaneous:	Sertraline	
Carbamazepine	**Paroxetine**	
Cisapride		
Dapsone		
Protease Inhibitors:		
Ritanovir		
Saquinavir		
Indanavir		

CYP3A has been implicated in the bioactivation of chemicals with examples being, aflatoxins and polycyclic aromatic hydrocarbons (Dresser *et al.*, 2000). Hence, there is a possibility that nevirapine might be bioactivated by this enzyme to a toxic metabolite, resulting in liver toxicity.

2.10 Methods for determining CYP3A activity

2.10.1 Probe drugs

Compounds that are selectively metabolised by specific P450 isoforms are also known as a marker drugs or probe drugs (Berthou, 2001). They have been used widely to provide information about enzyme activity, e.g. to assess effect of enzyme induction and inhibition (Okey, 1990). Many attempts have been made to develop probe drugs that can be safely administered to humans, so that the metabolism can be measured *in vivo*. It is however advantageous to conduct some studies *in vitro* using rat microsomes or human cell cultures because it obviates the ethical difficulties and, the environmental factors can be controlled.

Various substrate probes have been used to measure CYP3A activity *in vitro* and *in vivo*, examples include nifedipine, midazolam, testosterone-6β-hydroxylation and erythromycin (Wang *et al.*, 2000; Wang *et al.*, 1997; Ghosal *et al.*, 1996). Erythromycin demethylation is the most commonly used probe to measure CYP3A activity (Wrighton *et al.*, 1985a; Wrighton *et al.*, 1985b; Tu and Yang, 1983, Wang *et al.*, 1997) because it involves use of a simple spectrophotometer as opposed to the other probes, which use complex HPLC methods.

2.10.2 Erythromycin Demethylation Test

CYP3A4 is responsible for catalysing the metabolic conversion of erythromycin to its metabolites, formaldehyde and a demethylated compound in a ratio 1:1. Therefore formaldehyde has been used as an external standard for the determination of CYP3A activity *in vitro*. The determination is based on the Hantzsch reaction described by Nash (1953) where formaldehyde, in the presence of ammonium ions and acetyl acetone, yields a yellow coloured complex, diacetyl dihydrolutidine (DDL), that has an absorption maximum of 412 nm. This reaction is specific for formaldehyde.

PARTIII: IMMUNOCHEMICAL TECHNIQUES USED FOR PROTEIN DETECTION

Immunoassays and immunoblotting techniques have been widely used for detecting and analysing proteins. Although immunoassays were initially developed for the quantitation of hormones, its application has been expanded and includes the quantitation of several drugs and steroids. SDS-PAGE and the Western blotting techniques are the most often used for the detection and identification of specific CYP450 isoforms, and this is important to this study.

2.11 Immunoassays

Three immunoassays are commonly used, enzyme linked immunoassay (ELISA), radioimmunoassay (RIA) and fluorescent immunoassay (FIA). The basic principle of all immunoassays is the tendency of an antigen and its specific antibody to form an antigen-antibody-complex.

2.11.1 Radioimmunoassay (RIA)

RIA serves as one of the most essential techniques for the analysis of hormones, steroids and drugs in the clinical and biochemical environment. This technique involves the competitive binding of an unlabelled antigen and a radiolabelled antigen to the binding sites of an antibody (Ab). The unlabelled antigen diminishes the binding of labelled antigen present in the sample; the unlabelled antigen concentration can therefore be determined by using a standard (calibration) curve (McKee and McKee, 1994).

The advantages of this technique are that it can be used to assay any compound that can serve as an immunogen and that can be radiolabelled. RIA has high sensitivity as well as a high specificity (Bailey, 1984). This technique does however have disadvantages, the equipment and reagents are of a high cost, the reagents have a short shelf life and the duration of the assays are quite lengthy. This method can be radiologically hazardous and certain precautions have to be met (Burrin, 1994).

2.11.2 Enzyme-linked immunosorbent assay (ELISA)

This method is replacing RIA, as it is relatively inexpensive to operate and it lacks the radiological implications of RIA. Small laboratories are able to undertake this method because it is safer and less expensive than RIA (Burrin, 1994). ELISA is a very accurate and sensitive method of detecting antigens or haptens. It is based on antibody recognition of a particular antigenic epitope.

The competitive method makes use of a known amount of enzyme-labelled antigen with an unknown amount of unlabelled antigen; these are reacted with a specific antibody attached to a solid phase. A complex is formed and washed; the enzyme activity can be measured by the addition of a substrate. The difference between this value and the value of the sample lacking the unlabelled antigen is a measure of the concentration of unlabelled antigen. The disadvantage of this method however is that a different method may be required for each antigen.

The double antibody method uses an unknown antigen solution, which is reacted with a specific antibody that is attached to a solid phase. This complex is washed and enzyme-labelled antibody (secondary antibody) is applied. The amount of antigen present is in direct proportion to the measured enzyme activity (Gaastra, 1984; Burrin, 1994). The advantage of this method is that only one procedure is needed for the coupling of enzymes to an antibody. Table 2.6 compares the properties and characteristics of ELISA and RIA.

In general, ELISA involves the following steps (McKee and McKee, 1999)

- Antibody specific for an antigen is attached to an inert surface
- A small sample of the specimen is added, antigen-antibody binding commences
- A second, enzyme-linked antibody is added
- The unbound antibody is removed
- The enzyme produces a change in colour, which is proportional to the antigen concentration in the sample.

Table 2.6 Comparison of the properties of ELISA and RIA (Burrin, 1994).

	ELISA	RIA
Specificity	+	+
Sensitivity	+	+
Reproducibility	+	+
Cost	+	-
Reagent shelf life	+	-
Safety	+	-
Small laboratory	+	-

2.12 Electrophoresis and immunoblotting

There are three basic principles of the Western blot technique. First, proteins are separated by polyacrylamide gel electrophoresis (PAGE) and then, secondly, are transferred onto a membrane by electroblotting. Third, the individual protein of interest (CYP3A) is detected by immunodetection, using the appropriate antibodies and labelling methods (Egger and Bienz, 1994; Smith, 1984).

2.12.1 Electrophoresis

Electrophoresis is the process that uses an electric field through a matrix to separate charged molecules, such as proteins or nucleic acids. The speed of movement of different molecules is dependent on their shape, charge and size. Different types of electrophoresis techniques can be applied, with the most popular method being PAGE (polyacrylamide gel electrophoresis). Polyacrylamide is usually the medium of choice as it provides a stable medium that immobilises part of the system. The advantage of using polyacrylamide is the ease with which it is made and it can be made with a variety of pore sizes through which the proteins can move (Smith and Nicolas, 1983). This 3D matrix consists of polymers of acrylamide that are cross-linked together with the most common agent N,N' methylenebisacrylamide. Polymerization of the acrylamide is initiated by the addition of ammonium persulphate and N, N, N', N' - tetramethylenediamine (TEMED).

The separation of large proteins is better in lower % polyacrylamide gels whereas small proteins have a better separation in higher % gels. SDS-PAGE is the most

popular form of polyacrylamide gel electrophoresis (Smith and Nicolas, 1983); it involves denaturing of proteins in the presence of β-Mercaptoethanol and separates them according to their size (Fig. 2.7).

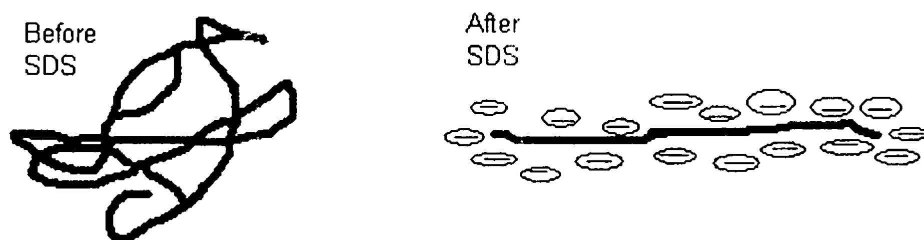


Figure 2.7 Anionic properties of SDS that uniformly provides proteins with a negative charge.

Usually urea-PAGE is mainly used for the separation of histone proteins, while non-denaturing or native gels are used to separate native proteins. The proteins are separated by their size and charge. Isoelectric focusing is a special form of native gel electrophoresis where a pH gradient is used to separate the proteins and they accumulate at their isoelectric point (Egger and Bienz, 1994). Since the scope of this script is confined to SDS-PAGE, isoelectric focusing will not be pursued any further.

The SDS-PAGE system most commonly used is the method described by Laemmli (1970), i.e. discontinuous electrophoresis. This system makes use of a stacking gel (pH 6.8) on top of the separating gel (pH 8.8). The wells in which the proteins are loaded are formed in the stacking gel. The advantage of using the stacking gel is that it provides sharp and straight bands, as the protein-SDS complex is concentrated in a tight band between the glycinate and chloride ions (Smith and Nicolas, 1983).

After the proteins have been separated by electrophoresis, electroblotting can be used to transfer proteins onto a membrane where they are immobilised and identified by means of antibodies (Switzer and Garrity, 1999).

2.12.2 Electrotransfer

Proteins can be transferred to a membrane either by diffusion or electroblotting. Transfer of proteins by diffusion is however not always complete as some proteins tend to remain in the gel (Gooderham, 1983). Electrotransfer involves application of

an electric field across the gel, forcing the proteins out of the gel and onto the membrane. This method is fast and virtually complete, providing a replica of the gel.

The different methods of electrophoresis and electroblotting require different buffers and membranes for the adequate transfer of proteins. A Polyvinylidene Difluoride (PVDF) membrane is often the membrane of choice as it has a high mechanical strength, high protein binding capacity and very good staining abilities. Table 2.7 provides a comparison of the properties of different membranes used in protein transfer. A gel sandwich is assembled, and placed into the transfer buffer, after which an electric field is applied (Fig. 2.8).

Table 2.7. Properties of membranes used for western blotting techniques (Egger and Bienz, 1994).

Membrane	Mechanical strength	Protein staining
Nitrocellulose	Poor	Good
Nylon	Good	Poor
PVDF	Good	Good

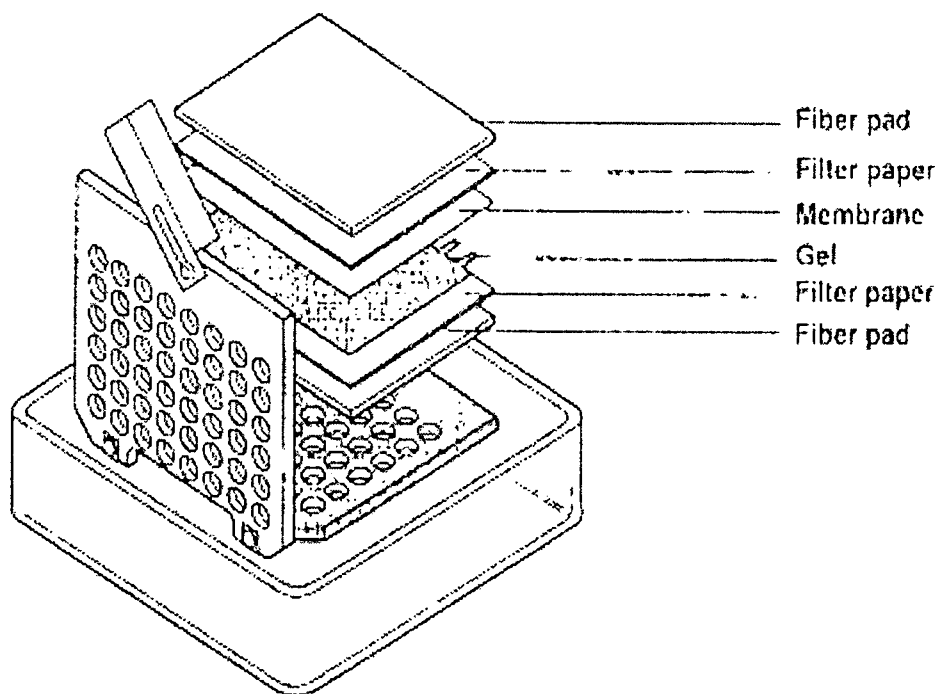


Figure 2.8 Assembly of the gel sandwich (Bio-Rad).

Once the proteins have been transferred to a membrane, various dyes can also be used to determine whether the transfer was successful. These include Amido Black, India ink, Ponceau, Colloidal gold and Coomassie blue. The nylon membrane is however not suitable for protein staining (Egger and Bienz, 1994).

Before the protein can be detected, free non-specific protein-binding sites on the membrane are blocked (Switzer and Garrity, 1999). Table 2.8 indicates different blocking agents that can be used to achieve this.

Table 2.8 Various blocking agents used to block non-specific sites on membranes (Egger and Bienz, 1994).

Blocking agent	Solution
Milk powder	5 % non-fat dried milk in 100mM Tris-HCl, pH 7.4, 150 mM NaCl
Tween-20	0.3 % Tween-20 in PBS
Ovalbumin / Gelatin	3 % Ovalbumin, 0.25 % gelatin in 100 mM Tris-HCl, pH 7.4

2.12.3 Immunoblotting / immunodetection

Immunoblotting involves the analysis of proteins transferred from a gel onto a membrane. A poly- or monoclonal antibody (Ab, MAb) directed against the protein of interest is used for the immunodetection of specific proteins. A secondary antibody coupled with an enzyme necessary for the production of a luminescent chemical reaction recognises the primary antibody and binds to it. The secondary antibody is often conjugated to an enzyme called horseradish peroxidase (HRP), the essential component necessary for the light-producing chemical reaction (Figs. 2.9 and 2.10).

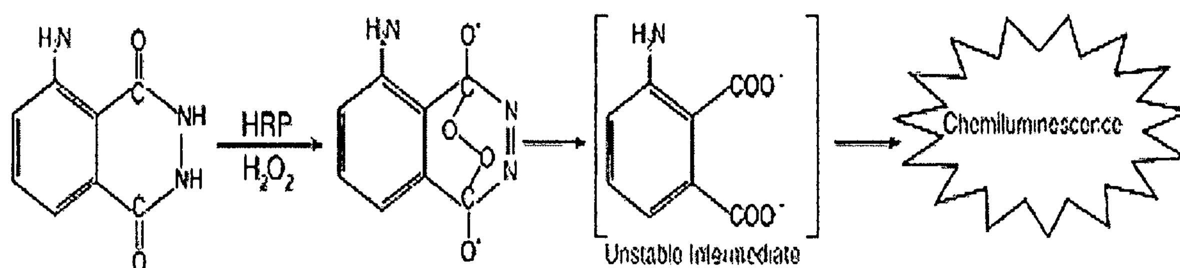


Figure 2.9 Production of chemiluminescence in the presence of horseradish peroxidase (http://www.roche-applied-science.com)

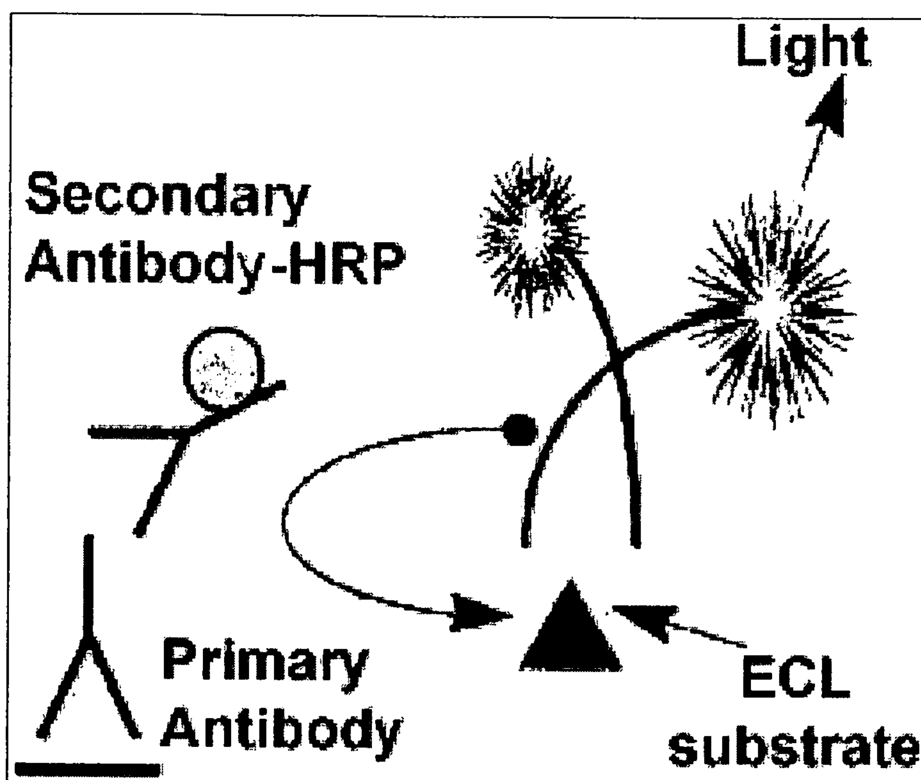


Figure 2.10 Schematic representation of the detection of proteins, using enzyme labelled antibodies (http://www.amershambiosciences.com/).

After incubations with antibodies, unbound antibody is removed from the membrane by washing the membrane with blocking buffer. Exposing the membrane to detection agents and using film for the development thereof detect the protein of interest (Egger and Bienz, 1994).

The overall process of protein detection therefore involves the separation of proteins, transferring them to a membrane and then detecting the protein by means of a specific antibody (Fig. 2.11).

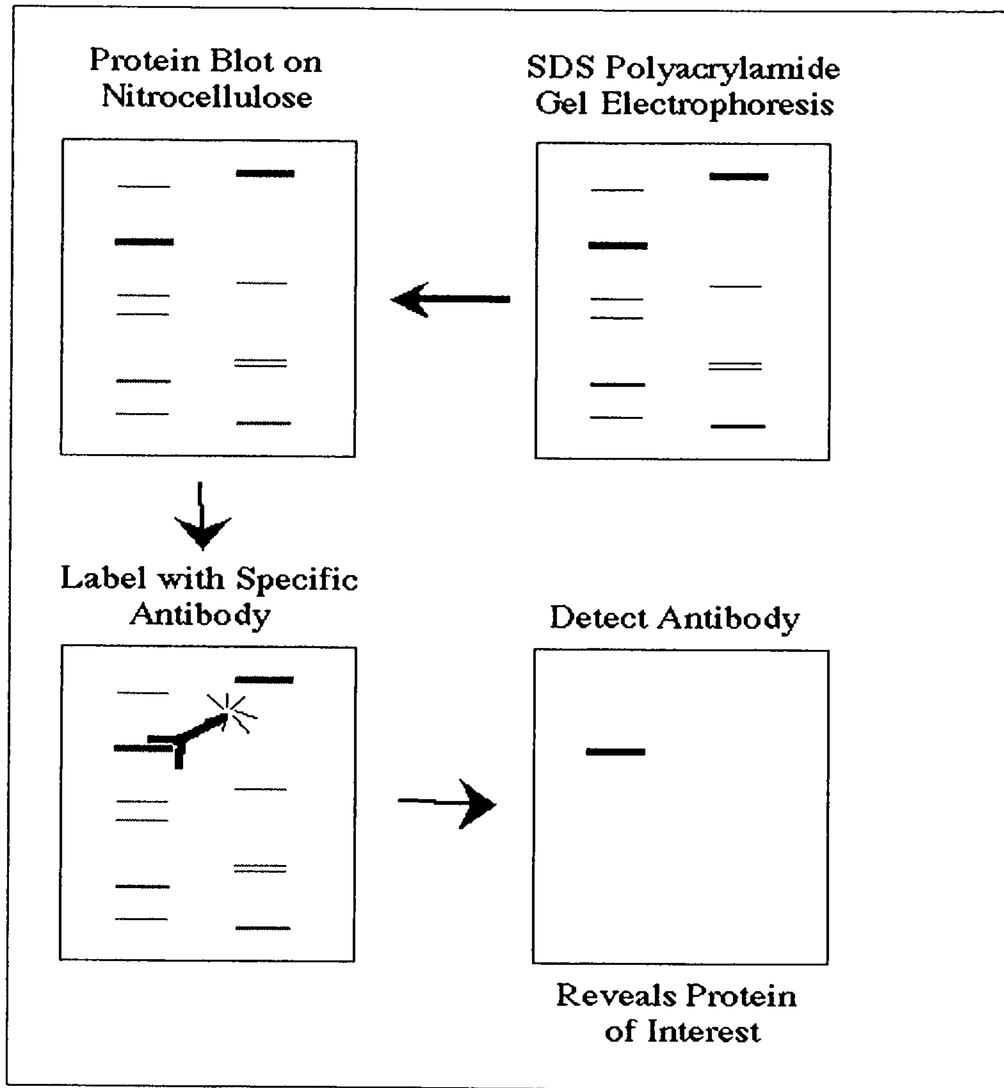


Figure 2.11 Schematic representation of the SDS-PAGE and western blotting technique, (http://www.bio.davidson.edu/Courses/genomics/method/Westernblot.html).

PART IV: DRUG INDUCED HEPATOTOXICITY

The liver has three major functions, firstly to maintain the constant nutrients within the blood (amino acids, glucose, fatty acids), secondly to dispose the body of unwanted constituents in the blood (ammonia, lactic acid, hormones, drugs and toxic substances) and third to produce plasma proteins (Peters, 1968). The liver is the most essential organ involved in the metabolism of exogenous and endogenous compounds and is one of the body's most vital organs. It behaves as a chemical factory by ridding the blood of toxic drugs and chemicals. This detoxifying organ excretes the metabolised products in the bile and is involved in practically all biochemical processes within the body. The functions of the liver are however affected as it is continuously exposed to chemicals, which could either lead to detoxification of the chemical or to the conversion of the chemical to a toxin.

2.13 Drug induced hepatotoxicity

The liver has a primary role in the metabolism of drugs as it serves as the major site for the entry of ingested foreign substances; therefore it is highly susceptible to the adverse effects of drug exposure (Mitchell and Jollows, 1975; Timbrell, 1983). Drug-induced hepatotoxicity remains a problem, as it is the most common reason for the withdrawal of approved drugs from the market (Lee, 2003).

Enzymes in the liver are responsible for converting a drug to a more polar compound so that it can be extracted into bile and removed from the body, this process is known as detoxification. This conversion does however not guarantee a less toxic or less active metabolite. Metabolites that are formed by drug metabolism may be less toxic or more toxic, less potent or more active (Rao, 1973).

The concepts of metabolic activation of drugs into carcinogenic substances was initially described by Miller and Miller (1966) and Magee and Barnes (1967), leading to the speculations made by Brodie (1971), that covalent binding of reactive metabolites may also lead to drug-induced tissue lesions.

2.13.1 Classification of Hepatotoxins

Hepatotoxins are classified into two categories according to their mechanism by which the agents produce hepatic injury. Firstly, some substances have an intrinsic property of injuring the liver (direct toxicity), whereas other substances damage the liver of susceptible hosts (Zimmerman, 1968; Rao, 1973; Grattagliano *et al.*, 2002). The first group is recognised by a high incidence of toxicity that is usually dose-dependent and the injury by this substance is reproduced in a variety of species. The function of the mitochondria and its enzymatic function is generally affected and disturbed. The second group depends on the host (idiosyncratic drug-induced reactions) rather than the substance itself (Zimmerman, 1968; Jaskiewicz, 1991). These reactions are unpredictable, don't appear to be dose dependent and are not reproducible. Hepatic injury is also categorised according to the type of liver injury, hepatocellular (cytotoxic) or cholestatic. Hepatocellular is recognised by necrosis or degeneration of the hepatic parenchyma, whereas cholestasis is characterised by jaundice and halted bile flow with the sparing of the hepatic parenchyma. Table 2.9 represents some examples of hepatic lesions due to drugs and toxins.

Tannic acid, 6-mercaptopurine and chlorphenothane are examples of pharmaceutical agents resulting in direct injury to hepatic cells. The lesions that are most often produced from such injury are fatty metamorphosis centrizonal necrosis.

It has been observed that the enzymatic pathways of the microsomal mixed-function oxidases have been responsible for the metabolic activation of some drugs. CYP450 enzymes in many instances cause the bioactivation of toxic precursors, they can convert non-toxic substances into hydroxylated derivatives and epoxides which are highly toxic compounds. This process forms Reactive Oxygen Species such as superoxide and hydrogen peroxide. These highly reactive compounds lead to the formation of free radicals that have a very high toxic potential (Pollak, 1996).

Table 2.9 A few examples of liver lesions due to drugs and toxins (Jaskiewicz, 1991 and Lee, 2003)

Lesion	Substance
Predictable	
Microvesicular fatty change	Valproate, tetracycline, didanosine
Phospholipidosis	Amiodarone
Necrosis	Paracetamol
Fibrosis	Methotrexate
Cholestasis	Contraceptive steroids
Venous occlusion	Pyrrolizidine alkaloids
Angiosarcoma	Vinylchloride, Thorium dioxide
Unpredictable	
Hepatitis	Isoniazid
Cholestasis	Chlorpromazine, eryhtromycin
Granuloma formation	Phenylbutazone, diltiazem, quinidine

Acetaminophen and furosemide are chemically stable compounds that are converted in the body to reactive alkylating and arylating agents. Covalent binding and hepatic necrosis were found to be dose-dependent in these two examples (Mitchell and Jollows, 1975, Park *et al.*, 1995). Pre-treatment of animals with inducers and inhibitors of metabolism altered the rate of hepatotoxin production and the severity of necrosis, as expected. The inhibitors of metabolism decreased the covalent binding of the toxic metabolite, which corresponded with the decreased hepatic necrosis (Mitchell and Jollows, 1975). Vessey (1990) described that when a drug generates a toxin, enzyme induction usually results in further toxicity and enzyme inhibition decreases the rate of metabolic activation, reducing the toxicity.

It is known that nevirapine therapy may result in serious hepatic events that can be life threatening. The biotransformation of nevirapine to its several metabolites is also well documented (Erickson *et al.*, 1999) but no association of the biotransformation and the hepatotoxicity has been made and the mechanism of induced hepatotoxicity is not known. The primary route of nevirapine biotransformation is via CYP3A and

therefore there is a need to investigate whether CYP3A has a role in nevirapine-induced hepatotoxicity.

PART V: DIAGNOSIS OF LIVER DISEASE

The diagnosis of liver disease requires a combined evaluation of the patient's clinical examination, radiological imaging and liver histology. Laboratory tests are used to assist in the diagnosis of liver disease and most often provides the initial requirement for further investigations of liver function (Kirsch, 1991).

2.14 Biochemical tests / Liver function tests (LFTs)

Liver function tests have provided a means of detecting the involvement of the liver by disease, evaluating the degree and type of impairment of liver function, differentially diagnosing liver diseases and following the course of the liver disease (Rawnsley, 1968; Wilkonson, 1968; Timbrell, 1983). The biochemical tests are the most widely used tests to determine liver dysfunction (Kirsch, 1991). They provide useful information about the type of liver damage and they are particularly useful in differentiating between hepatocellular necrosis and cholestasis.

Liver function tests commonly include the measurement of several enzymes that help the body metabolise substances. Enzymes that are often measured include gamma-glutamyl transferase (GGT), alanine aminotransferase (ALT), aspartate aminotransferase (AST) and alkaline phosphatase (ALP). LFT's may also include the measurement of prothrombin time as well as bilirubin. However, serum aminotransferases are most often measured and elevations of these enzymes in circulation are indicative of liver disease (Kirsch, 1991; Stolz and Kaplowitz, 1990). However, Liver function tests, alone are poor indicators of the prognosis and severity of liver disease (Kirsch, 1991).

2.14.1 Important liver enzymes commonly measured

Serum transaminases, aspartate aminotransferase (AST) and alanine aminotransferase (ALT) activities are markers for hepatocelluar necrosis, whereas alkaline phosphatase (ALP) serves as a marker for cholestasis. Table 2.10 indicates many other enzymes that are also markers of liver disease.

Table 2.10 Serum markers for liver disease (Kirsch, 1991; Stolz and Kaplowitz, 1990)

Liver disease and abnormalities	Hepatocellular necrosis	Cholestasis	Metabolism and synthetic function
Markers	Serum transaminases Lactate dehydrogenase	Alkaline phosphatase Gamma-Glutamyl Transpeptidase Leucine aminopeptidase	Urea synthesis Albumin Clotting proteins Immunoglobulins of liver disease Cholesterol Lipids Lipoproteins

2.14.2 Serum Transaminases

2.14.2.1 Alanine aminotransferase (ALT)

ALT is found predominantly in the liver (Stolz and Kaplowitz, 1990) and is produced within the cytosol of the hepatocytes. The levels in the blood are increased due to hepatocyte damage or cell death. The damaged cells result in ALT leakage out into the bloodstream. Although ALT is present in other tissues, the highest concentration is in the liver and therefore elevation of this enzyme within the bloodstream is the most specific indicator of liver injury today.

2.14.2.2 Aspartate aminotransferase (AST)

AST is found in many different tissues; the liver, heart, kidney, skeletal muscle and brain, making elevations in the blood slightly less specific for liver injury than ALT. Four fifths of liver AST is found in the mitochondria, while one fifth is present in the

cytosol. This enzyme is very similar to ALT and in the case of liver toxicity, elevations of AST usually parallel those of ALT (Götz, 1980).

2.14.2.3 Alkaline phosphatase (ALP)

ALP isoenzymes are widely distributed in human tissue, e.g. (bone, kidney, intestine, placenta and liver), therefore are not as specific for liver disease (Kirsch, 1991). In liver disease, the highest activity of this enzyme is found in patients with cholestasis or hepatic carcinoma (Stolz and Kaplowitz, 1990; Götz, 1980). Increased serum ALP activity is also found in some patients with cirrhosis or chronic hepatitis.

2.15 Pathologic diagnosis of Hepatic disease

Histopathology is essential for comprehending the pathogenesis and classification of liver disease.

The histological manifestations of the hepatocellular necrosis (cytotoxic form) of liver injury include many lesions; zonal necrosis, fatty metamorphosis, diffuse necrosis and degeneration. Hepatocellular injury is characterised by necrosis and degeneration of the hepatic parenchyma. Zonal necrosis is termed as the predilection of hepatocyte necrosis for a specific microanatomic region of the lobule or acinus, often referred to as centrilobular (zone 3) necrosis (Vessey, 1990).

The histological manifestations of cholestatic injury include an intact parenchyma with prominent bile casts in the canaliculi (Zimmerman, 1968). Vessey (1990) and Jaskiewicz (1991) described the histological manifestations of cholestasis as the presence of bile in the liver tissue, reflecting arrested bile flow.

In general, whenever there is a fatal liver problem, this is captured by histopathology studies. This is also important in any study of hepatotoxicity as intended to be done here.

CHAPTER 3

OBSERVATIONS FROM REVIEW, HYPOTHESIS AND OBJECTIVES

3.1 Observations from review

In summary, it was observed that:

3.1.1 Nevirapine induced hepatotoxicity commonly occurred from 2-12 weeks of therapy.

3.1.2 The major enzyme involved in the metabolism of nevirapine is CYP3A.

3.1.3 Nevirapine is an autoinducer and induces CYP3A and CYP2B6

3.1.4 No association between nevirapine-induced hepatotoxicity and the CYP3A enzymatic pathway has been made.

3.2 Hypothesis

'CYP3A has a role in nevirapine-induced hepatotoxicity'

3.3 Objectives

To investigate the role of CYP3A in nevirapine induced hepatotoxicity by:

3.3.1 Determining CYP3A activity in rat microsomes.

3.3.2 Establishing the optimum time for CYP3A enzyme induction in rats using nevirapine and dexamethasone as inducers.

3.3.3 Determining the role of CYP3A inhibitors in preventing nevirapine-induced hepatotoxicity.

CHAPTER 4

THE MEASUREMENT OF RAT MICROSOMAL CYP3A ACTIVITY IN VITRO

4.1 Introduction

To achieve the aim of this study, knowledge of CYP3A activity in the test animal is mandatory. Therefore, in this chapter, procedures for determining rat CYP3A activity *in vitro* are described. First, animal experiments involving the removal of rat livers, the extraction of microsomes and quantitation of microsomal proteins are described. This is followed by a chronological description of events in the assay for microsomal CYP3A activity in normal rats, using the erythromycin demethylation test (Wrighton *et al.,* 1985b).

4.2 Reagents

Di-sodium hydrogen orthophosphate dihydrate, sodium dihydrogen orthophosphate dihydrate, potassium dihydrogen phosphate, di-potassium hydrogen phosphate, diethyl ether, ammonium acetate, trichloroacetic acid, dimethylsulphoxide (DMSO), barium hydroxide and glacial acetic acid were purchased from Merck (Darmstadt, Germany). Ethanol and zinc sulphate were purchased from BDH Laboratory supplies (England, U.K.). Erythromycin, magnesium chloride, semicarbazide, TrismaBase [tris(hydroxymethylaminomethane)], ethylenediaminetetraacetic acid (EDTA), formaldehyde, potassium chloride, D-glucose-6-phosphate monosodium salt, ß-Nicotinamide adenine dinucleotide phosphate sodium salt and glucose-6-phosphate dehydrogenase were obtained from Sigma Chemical Co. (St Louis, MO USA). A Protein Assay ESL Kit (Roche Diagnostics Corporation, Indianapolis, IN, USA) was used to measure the microsomal protein concentration. This Kit consisted of Reagent A (alkaline copper tartrate solution), Reagent B (ascorbic acid, bathocuproine disulfonic acid solution) and an external standard *viz.* BSA (bovine serum albumin) protein standard (2 mg/ml) in 0.9% NaCl.

4.2.1 Special buffers and prepared reagents

Buffer 1 consisted of TrisOac (0.1 M at pH = 7.4), KCl (0.1 M), EDTA (1mM) and butylated hydroxytoluene (25 μM). Buffer 3 consisted of TrisOac (10 mM, pH = 7.4), EDTA (1 mM) and glycerol (20%). The Nash reagent was composed of ammonium acetate, glacial acetic acid and acetyl acetone in final concentrations of 2 M, 0.05 M and 0.02 M respectively.

4.3 Apparatus

A cold glass mortar and pestle were used to grind the rat liver to pieces and an electric grinder (Ultra-turrax, Janke and Kunkel) was used to homogenise the liver suspensions. A Beckman JA 20 (rotor J2-21) centrifuge was used to perform the first centrifugation of the microsomal preparation and a Beckman L8-70M ultracentrifuge (SW28 rotor) was used for the final ultracentrifugation. UV absorption was measured using an Ultrospec II, LKB Boichem spectrophotometer. A shaking waterbath (Labcon) was used for the microsomal incubations and an eppendorf centrifuge (5810 R) was used to sediment the proteins.

4.4 Animals

Male Sprague-Dawley rats (Animal House, University of the Free State) weighing 300 – 350 g were used and the animal Ethics Committee approved the study.

4.5 Part I: Animal experiments

4.5.1 Methods

4.5.1.1 Surgical procedure and blood collection

Rats were anaesthetised with diethyl ether and the abdomen was opened. The abdominal contents were gently pulled aside and blood (5 ml) was drawn from the abdominal vein in heparinised tubes and placed immediately on ice. The blood samples were centrifuged within 15 min at 3200 *g* for 10 min and the supernatant (plasma) was sent for liver function tests. The rat was bled to remove as much blood as possible, after which, the liver was excised from its supporting structures, washed in ice-cold 1.15 % KCl, placed in liquid nitrogen and stored at -80 °C until use.

4.5.1.2 *Microsomal preparation*

Liver pieces weighing approximately 4 g were cut into small pieces after thawing. Buffer 1(4 X volume) was added to the pieces and they were ground using the cold glass mortar and pestle. The liver suspension was homogenised further with an electric grinder at speed 2 for 30 s. The homogenised mixture was centrifuged (JA 20 rotor, Beckman centrifuge) at 10,000 *g* for 20 min at 4 °C. The fatty layer was removed and the supernatant (microsomal fraction) was transferred to ultracentrifugation tubes. The remaining pellet was washed with Buffer 1(2 x volume) and centrifuged as above. The resulting supernatant was ultracentrifuged (SW28 rotor, Beckman L8-70M) at 4 °C for I hour at 100 000 *g*. The supernatant was discarded and the remaining pellet was re-suspended in Buffer 3. The suspension was aliquoted and stored at – 80 °C until further use.

Aliquots of this stock microsomal solution were used to measure microsomal protein concentration as described below. Microsomal protein concentration is used as a standard measure for microsomes used in reaction mixtures.

4.5.1.3 *Determination of microsomal protein concentration*

Calibration: Protein concentrations were measured using the Protein Assay ESL Kit (Roche Diagnostics Corporation) according to the protocol supplied with the Kit. Briefly, Reagent A and Reagent B were brought to room temperature. Using bovine serum albumin (BSA), a series of standards in the concentration range of 0 - 750 μg/ml (i.e., 0, 50, 100, 300, 500 and 750 μg/ml) in a final volume of 400 μl were prepared by diluting the BSA protein standard with deionised water.

To each appropriate micro cuvette was added 100 μl of Reagent A and 50 μl of the prepared standard. One millilitre of Reagent B was added to each cuvette and the absorbance was measured at 30 s at 485 nm. A calibration curve was then constructed by plotting the absorbance of the standards versus their concentrations.

Determination of protein content in microsomal samples: To determine the protein concentration in the unknown samples, 20 μl of the microsomal sample was diluted to a final volume of 400 μl with deionised water and the calibration procedure

described above was followed. A calibration was performed each time the protein concentration of a batch of microsomes had to be determined. The linear equation from the calibration curve was used to derive the protein concentration of the microsomes.

4.5.2 Results

Table 4.1 represents data used to construct a calibration curve. The calibration curve proved to be linear with a regression equation of $y = -0.0009x + 1.0054$ and a correlation coefficient of 0.9896 (Fig. 4.1).

Table 4.1 Absorbance values of the corresponding standards.

BSA (μg/ml)	Abs 412 nm
0	0.996
50	0.943
100	0.945
300	0.71
500	0.533
750	0.47

BSA = Bovine Serum Albumin

Abs = Absorbance

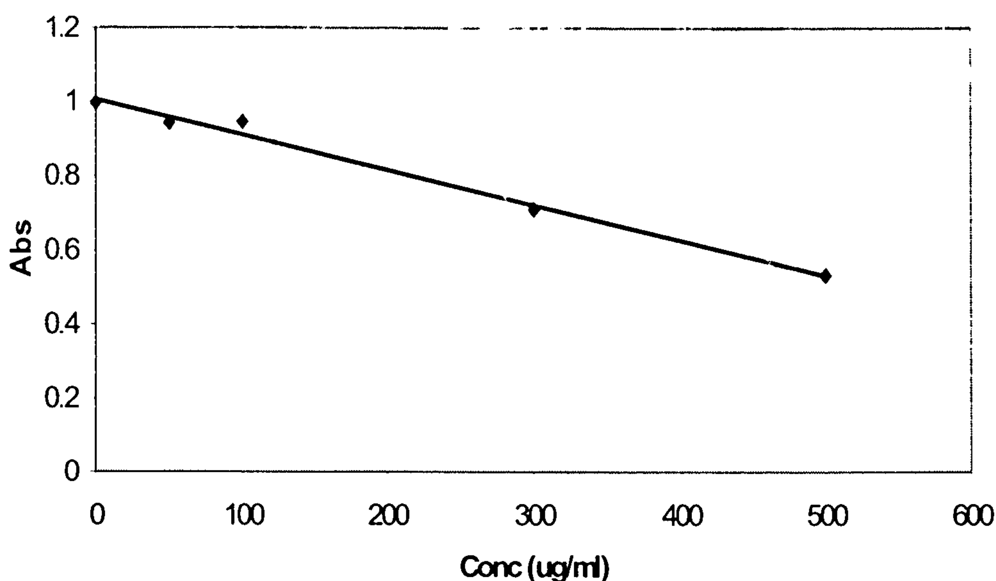


Figure 4.1 Calibration curve of BSA standards, y = -0.009x + 1.0054, r^2 = 0.9896.

4.6 PART II: Unexpected low CYP3A activity

Success of this experiment requires optimum CYP3A activity in the test sample. As such, CYP3A activity in microsomes was tested using the conditions as reported in the literature (i.e., protein content, time of incubation and substrate concentration), Wrighton *et al.* (1985b).

4.6.1 Methods

4.6.1.1 Reaction conditions for CYP3A assay

The method described by Wrighton *et al* (1985b) was used with the following modifications. Microsomal proteins were diluted in 0.1 M sodium phosphate buffer (pH 7.4) to a desired protein concentration. Then erythromycin, magnesium chloride and semicarbazide were added to final concentrations of 5 mM, 160 μM and 320 μM respectively. This reaction mixture was pre-incubated for 10 min after which the reaction was initiated by the addition of the NADPH re-generating system. The final concentrations of glucose-6-phoshate, NADP and glucose-6-phosphate dehydrogenase were, 10 mM, 0.5 mM and 0.05 units respectively. The total reaction volume was 0.5 ml. The reaction was incubated at 37 °C for 10 min and quenched with 200 μl of 2.5 % zinc sulphate followed by 200 μl of saturated barium hydroxide.

4.6.1.2 Sample preparation

To sediment the proteins, the tubes were centrifuged at 3200 *g* for 10 min, 650 μl of the supernatant was transferred to another tube and mixed with an equal volume of Nash reagent. The mixture was incubated at 37 °C for 30 min and then cooled to room temperature. The samples were analysed by measuring absorbance at 412 nm using a spectrophotometer.

4.6.1.3 Troubleshooting

Unfortunately, poor CYP3A activity was observed whereby some reactions did not even produce any formaldehyde. It was therefore necessary to improve the reaction conditions and to eliminate those conditions responsible for the poor metabolite productivity (troubleshooting).

- Increased erythromycin concentration (1)

It was decided to increase the final erythromycin concentration to 10 mM and to use a fresh Nash reagent. No difference or improvement in the metabolite production was observed under these conditions.

- Varying times of incubation

Varying times of incubation were tried with a new batch of microsomes, 0.2 mg per reaction, these reactions showed a slight increase in metabolite production, but the assay was not reproducible.

- Further increase in erythromycin concentration (2)

The substrate concentration was further increased to a final concentration of 30 mM; this did not cause any improvement in metabolite production. It was therefore necessary to determine whether other factors were responsible for the unsuccessful production of formaldehyde.

- Change in pH

After the sample preparation, the pH of the samples were adjusted to 4.5 and then incubated with the Nash reagent, the results were worse than those previously obtained.

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- Varying stop solution

According to the method described by Panvera Corporation, trichloroacetic acid was used to quench the reaction, this was tried but these conditions did however not improve the reaction and poor metabolite production was still a problem.

- Decreased erythromycin concentration (3)

Erythromycin (EM) serves as a substrate and an inhibitor of CYP3A, it was thought that increasing EM would lead to a reduction in CYP3A activity and therefore it was decided to decrease the amount of substrate (erythromycin) within the reaction. The protein content in the reaction was increased to 0.5 mg with varying final concentrations of EM (0.5 mM, 1 mM and 2 mM) and the microsomes were diluted using a potassium phosphate buffer (pH 7.4). No difference was observed between these reactions of varying EM concentrations and metabolite production remained to be very poor.

- Varying temperature

The Hantzch reaction was performed at 37 °C for 60 min and at 50 °C for 30 min, this variation in time and temperature did not affect the results that were previously obtained.

- New batch of erythromycin

A different type of erythromycin (Erythromycin lactobionate, Abbot) under the same reaction conditions was used to determine whether the original EM (Sigma) had a role in the poor production of metabolite but no difference was noted.

- Change in erythromycin solvent

Erythromycin was dissolved in alcohol and it was thought that the alcohol could be affecting the activity of the enzyme or perhaps precipitating the proteins before the reaction was started. DMSO was used as the solvent.

- Use of EDTA instead of semicarbazide

According to the method described by Wang *et al.* (1997) semicarbazide was not included in the reaction conditions, this was replaced with EDTA. EDTA was added

to the reaction in a final concentration of 0.2 mM and $MgCl_2$ in a final concentration of 4 mM. Low amount of formaldehyde was produced and this reaction remained unacceptable for determining CYP3A activity.

- Increased protein concentration

The protein content was increased to 0.8 mg per reaction with double the amount of co-factors in the NADPH-generating system, EM was added in a final concentration of 5 mM. The amount of metabolite production was not influenced and no difference was observed on comparison with previous findings described above.

4.6.1.4 Comment

The results of this method remained unchanged and poor throughout the optimisation process. From the conditions described above, it was clearly seen that all the reagents that could be responsible for the poor production of metabolite have been eliminated. It was therefore decided to determine whether the CYP3A enzyme within the rat microsomes was responsible for the poor yield in metabolite. Male SD rats were used throughout and there was a possibility that the way in which they were bred and maintained could have had an effect on the amount of CYP3A expressed within the liver. It was therefore decided to induce CYP3A using a known inducer (dexamethasone) and then to compare the amount of metabolite produced with the untreated rats. This would determine whether the poor expression of CYP3A in rat liver microsomes was responsible for the lack of metabolite production or if it was due to the reaction conditions of the assay described above.

Dexamethasone (50 mg/kg/day) was administered intraperitoneally to the rats for a period of 3 days. The animals were sacrificed on the fourth day and microsomes were extracted from the excised livers (Part I). The activity of CYP3A was determined as before.

4.6.2 Results

Table 4.2 shows that the activity of CYP3A in uninduced rats was very poor while the activity in the dexamethasone treated rats was high, thus proving that the reaction conditions were not responsible for the poor yield in metabolite, but that CYP3A needed to be induced with dexamethasone to obtain an active enzyme (Fig. 4.2).

Table 4.2 The activity of microsomal CYP3A (nmol/min/mg protein) in untreated and dexamethasone pre-treated rats.

Rat	Untreated (nmol/min/mg protein)	Dexamethasone (nmol/min/mg protein)
1	0.14	9.89
2	1.09	16.28
3	0.62	7.93
4	1.02	7.17
5	0.07	10.70
Average	0.59	10.39
± SD	0.48	3.59

P = 0.0079, considered very significant (Mann-Whitney U Test, 0.05 level of significance).

SD = standard of deviation

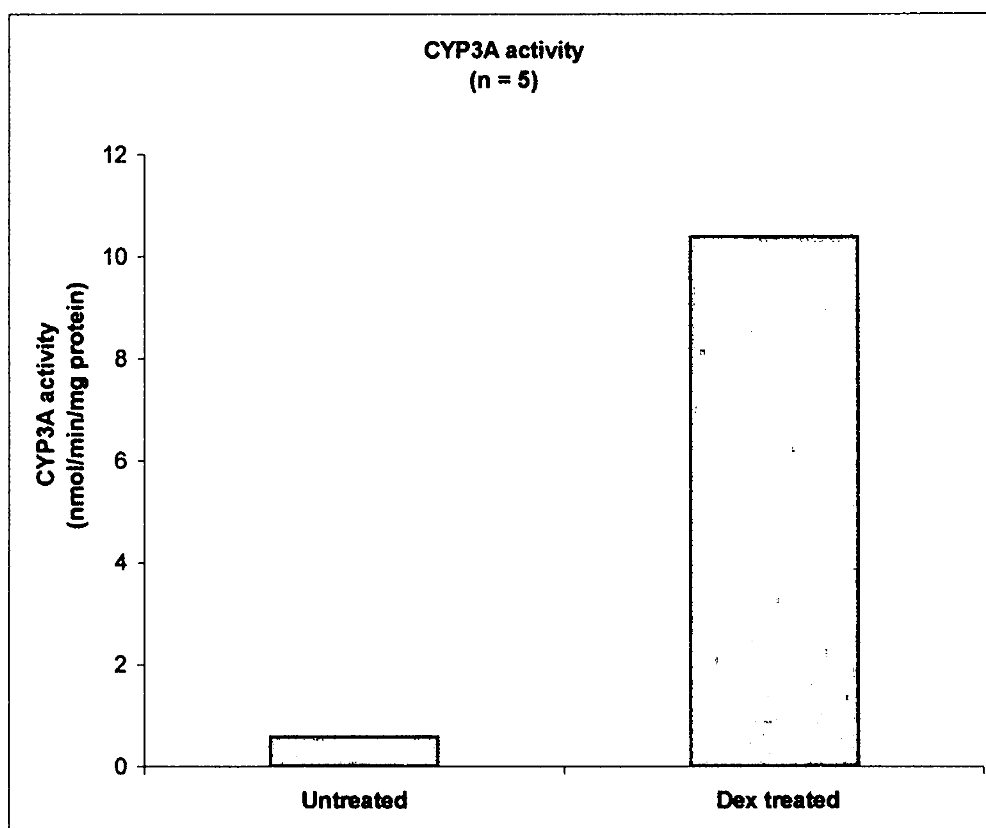


Figure 4.2 The average activity of microsomal CYP3A in untreated and dexamethasone treated rats.

4.7 Discussion

It has been proven that the reagents involved in the assay for the measurement of CYP3A activity were not responsible for the poor yield of metabolite. This poor yield was probably due to the lack of CYP3A activity in the untreated rats because the dexamethasone treated rats showed a marked improvement in the activity of CYP3A. Interestingly, treatment with dexamethasone was not required in previous reports (Wrighton *et al.*, 1985a), indicating that there could be variations in rat CYP3A expression. This implies that one cannot assume a uniform expression of CYP3A even in the same species.

After pre-treatment with dexamethasone, optimum CYP3A activity was achieved and this enabled further evaluation and setting up the assay for CYP3A activity, (i.e. the erythromycin demethylation assay as described in the next chapter.

CHAPTER 5

STANDARDISATION OF REACTION CONDITIONS FOR THE ASSAY OF RAT MICROSOMAL CYP3A ACTIVITY

5.1 Introduction

To study enzyme activity *in vitro,* the reaction conditions have to be standardised to a point where optimum enzyme activity is attained. Here, the method described in Chapter 4 will be used to set the standard reaction conditions for the *in vitro* assay of rat microsomal CYP3A activity. This involved determining the concentration of microsomal protein content, substrate (erythromycin) and time of incubation at which optimum enzyme activity is attained.

5.2 Reagents

Potassium dihydrogen phosphate, di-potassium hydrogen phosphate, diethyl ether, ammonium acetate, dimethylsulphoxide (DMSO), barium hydroxide and glacial acetic acid were purchased from Merck (Darmstadt, Germany). Zinc sulphate was purchased from BDH Laboratory supplies (England, U.K.). Dexamethasone, erythromycin, magnesium chloride, ethylenediaminetetraacetic acid (EDTA), formaldehyde, D-glucose-6-phosphate monosodium salt, ß-Nicotinamide adenine dinucleotide phosphate sodium salt and glucose-6-phosphate dehydrogenase were obtained from Sigma Chemical Co. (St Louis, MO USA)

5.3 Apparatus

An Ultrospec II, LKB Boichem spectrophotometer was used to measure the UV absorption of formaldehyde and an eppendorf centrifuge (5810 R) was used to sediment proteins of the microsomal assay. A shaking waterbath (Labcon) was used for the incubation of the microsomal enzyme assays.

5.4 Methods

5.4.1 Assay for CYP3A activity *in vitro.*

All procedures were performed on ice. Since CYP3A activity in normal rats was very low, the microsomes used in this experiment were from dexamethasone treated rats

as described in chapter 4. Aliquots of microsomal suspensions were diluted with 0.1 M potassium phosphate buffer (pH7.4) to which EDTA and $MgCl_2$ were added to final concentrations of 0.2 mM and 4 mM respectively. Erythromycin was added to a final concentration of 5 mM and the reaction was pre-incubated for 10 min at 37 °C in a shaking waterbath after which the reaction was initiated by the addition of the NADPH-generating system (consisting of final concentrations of 0.05 units glucose-6-phosphate dehydrogenase, 10 mM glucose-6-phosphate and 0.5 mM NADP), with a total reaction volume amounting to 0.5 ml. The reaction was incubated further for 10 min and halted by the addition of 200 µl of 2.5 % $ZnSO_4$ followed by 200 µl of saturated $Ba(OH)_2$. Proteins were separated by centrifugation for 10 min at 3200 *g*. An equal volume of the supernatant (650 µl) was added to an equal volume of Nash reagent (650 µl), this mixture was incubated at 40 °C for 60 min and then cooled down to room temperature. The amount of formaldehyde produced was determined with a spectrophotometer at 412 nm.

5.4.2 Calibration for the determination of formaldehyde content in microsomal solutions.

Since formaldehyde is the product of erythromycin demethylation by CYP3A, it was used as the external standard to construct a calibration curve. Inactivated (boiled) microsomes were used to perform the calibrations. The same conditions described above were followed with the inclusion of formaldehyde as the external standard. Formaldehyde standard solutions were prepared in microsomal solutions to obtain the following concentrations; 50, 100, 150, 200 and 250 nmol/ml. These standards were treated as described above after which absorbance was read at 412 nm. This procedure was performed on five different days to ascertain reproducibility and linearity. Data (absorbance vs. concentration) was analysed by linear regression. A calibration curve was performed with each batch.

5.4.3 Methods for standardisation of reaction conditions

5.4.3.1 Microsomal protein content.

The assay procedure described in (5.4.1) was used but with varying microsomal concentrations as follows; 0.1, 0.2, 0.3, 0.4, 0.5 and 0.6 mg protein (appendix A-1).

5.4.3.2 Time of incubation.

The assay procedure described in (5.4.1) was used with 0.4 mg of microsomal protein (0.8 mg/ml) in each reaction. The reactions were incubated for various time periods; 2.5, 5, 7, 10 and 15 min (appendix A-2).

5.4.3.3 Substrate concentration.

The assay procedure described in (5.4.1) was used with 0.4 mg of microsomal protein (0.8 mg/ml) in each reaction. Erythromycin (the substrate) was added in various final concentrations (1, 2, 5, 10 and 20 mM) and the reactions were incubated for 10 min (appendix A-3).

5.5 Results

5.5.1 Formaldehyde Calibration results

Table 5.1 shows the calibration data on the five different days, while Figure 5.1 illustrates the average absorbance values. The average linear regression equation was y = 0.002x + 0.0118, with a regression coefficient (r^2) of 0.997 (n = 5 days). Overall, CV% was less than 15 %.

Table 5.1 Formaldehyde calibrations performed on different days.

CH_2O (nmol/ml)	Abs Cal 1	Abs Cal 2	Abs Cal 3	Abs Cal 4	Abs Cal 5	Mean Abs	± SD	CV %
0	0	0	0	0	0	0.000	0.000	0.000
50	0.138	0.114	0.143	0.141	0.122	0.132	0.013	9.770
100	0.232	0.22	0.228	0.183	0.21	0.215	0.020	9.117
150	0.349	0.32	0.329	0.251	0.313	0.312	0.037	11.808
200	0.481	0.357	0.426	0.352	0.43	0.409	0.054	13.311
250	0.601	0.504	0.525	0.448	0.539	0.523	0.056	10.607

CH_2O = Formaldehyde

Abs = Net Absorbance

SD = Standard of Deviation

CV = Coefficient of variation

Cal = Calibration

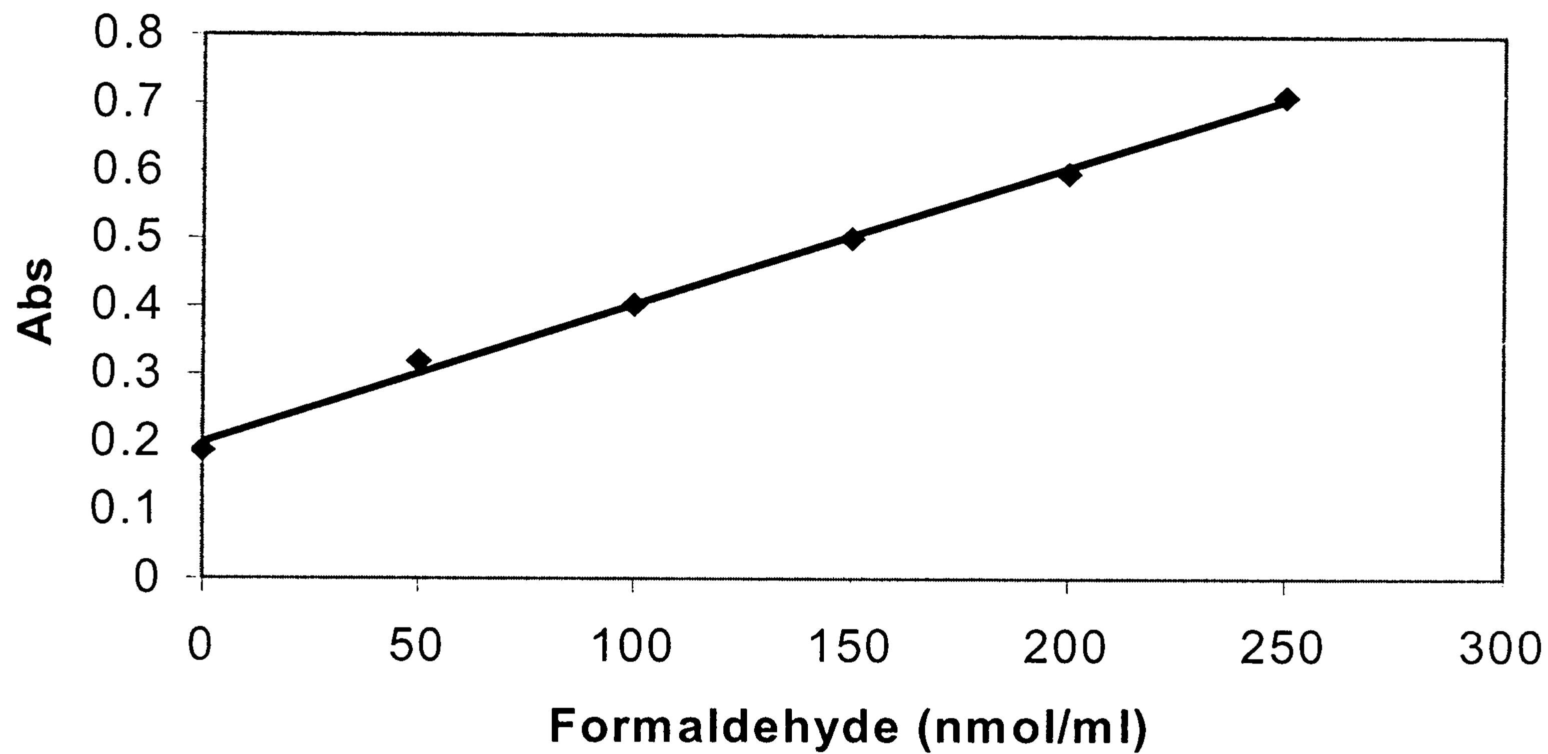


Figure 5.1 The average calibrations of formaldehyde, the product of erythromycin demethylation.

5.5.2 Results for the standardisation of reaction conditions

5.5.2.1 Microsomal protein

Table 5.2 and Figure 5.2 illustrate the effect of different protein concentrations on the activity of CYP3A. Metabolite production (enzyme activity) was proportional to the concentration of microsomal protein in the reaction mixture, implying that CYP3A activity was linear throughout the protein concentration range evaluated. It was therefore decided to use 0.4 mg as the standard amount of microsomal protein content in each reaction (0.8 mg/ml) as it has been used elsewhere.

Note: calibration data for this batch is shown in appendix A-4. The calibration curve was linear with a regression equation of $y = 0.0013x + 0.0089$ and r^2 of 0.994.

Table 5.2 CYP3A activity at various protein concentrations.

Protein	CYP3A activity (nmol/min/mg)
0.1	3.423
0.2	6.837
0.3	8.471
0.4	11.404
0.5	14.288
0.6	16.788

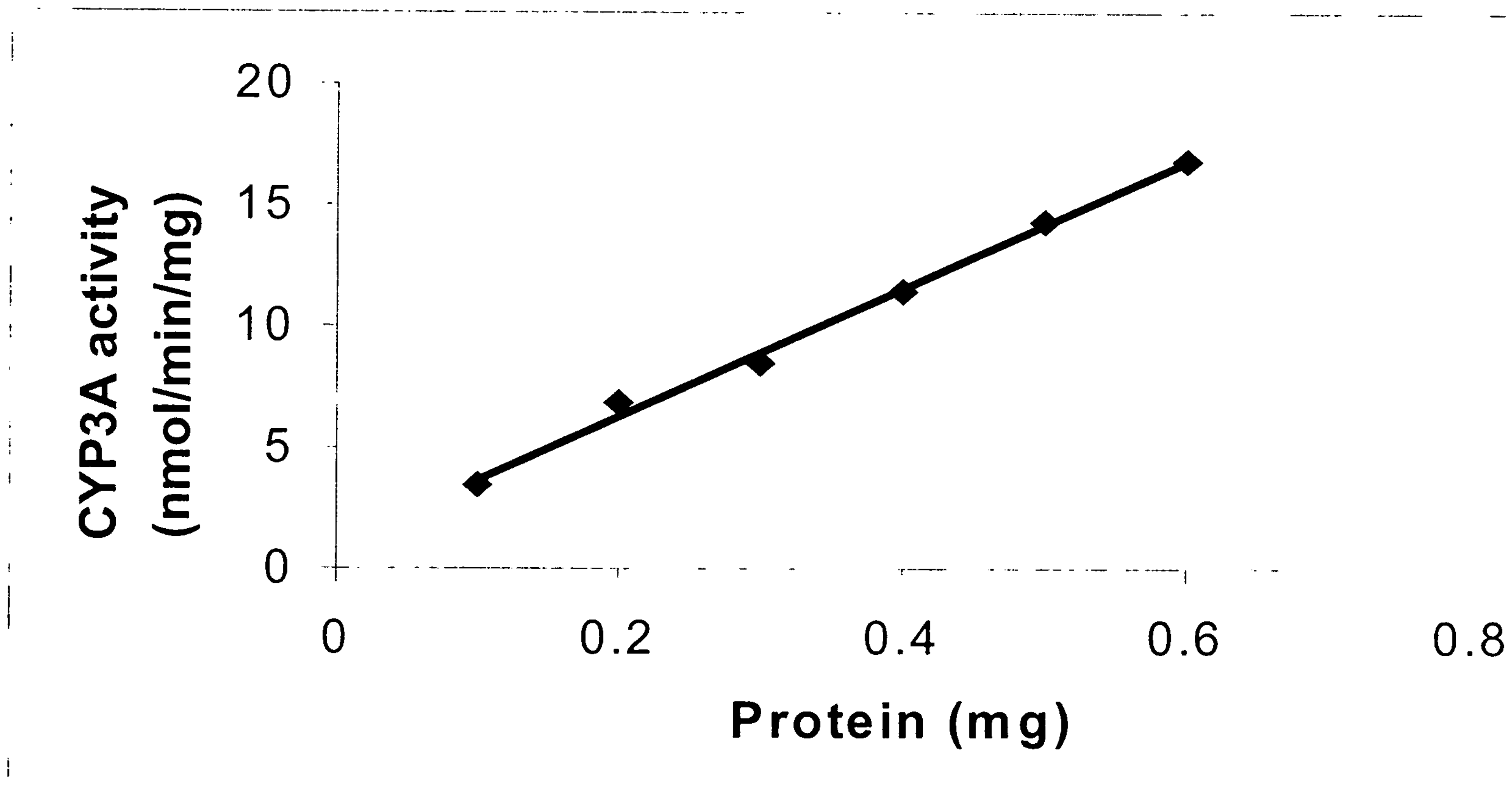


Figure 5.2. A plot of CYP3A activity at varying protein concentrations

5.5.2.2 Time of incubation.

Table 5.3 and Figure 5.3 shows the effect of different incubation times on the amount of metabolite (formaldehyde) produced. The amount of metabolite produced was proportional to the respective time periods of incubation, implying that CYP3A activity was linear throughout this time range. It was therefore decided to use 10 min as the standard time of incubation, as this has been used in previous methods (Wrighton *et al.*, 1985b) and it is very convenient for the experimental purposes.

Note: Calibration data from which the amount of formaldehyde produced was derived, is shown in appendix A-5. The curve was linear with the equation $y = 0.0014x + 0.0058$ and r^2 of 0.998.

Table 5.3 Amount of metabolite (formaldehyde) produced at various incubation times.

Time of incubation (min)	Metabolite produced (nmol)
2.5	12.93
5	24.71
7	28.82
10	38.64
15	56.32

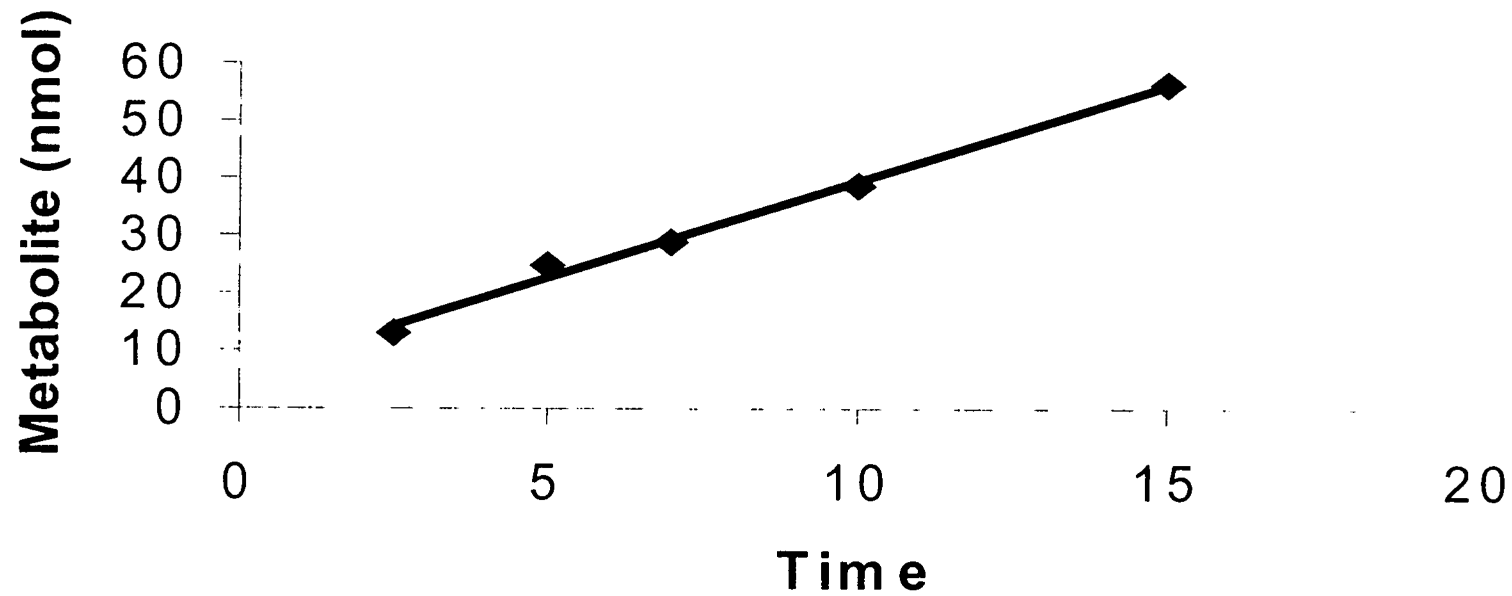


Figure 5.3 A plot of the amount of metabolite produced at varying times of incubation

5.5.2.3 Substrate concentration

Table 5.4 and Figure 5.4 shows the effect of varying substrate (erythromycin) concentrations on the activity of CYP3A. At low substrate concentrations, the rate of the reaction was proportional to the substrate (erythromycin) concentration. However at sufficiently high substrate concentrations, the enzyme became saturated, resulting in a constant reaction rate. It was decided to use 5 mM as the standard amount of substrate used in the reaction, as optimum enzyme activity was attained here. From the graph, it was noted that the Km was 0.65 mM (Km = [substrate] at Vmax/2).

Note: The calibration curve was linear with a regression equation of $y = 0.0014x + 0.097$ and r^2 of 0.99 (appendix A-6).

Table 5.4 CYP3A activity at varying substrate (erythromycin) concentrations.

Erythromycin (mM)	CYP3A activity (nmol/min/mg)
1	6.723
2	7.929
5	9.580
10	10.964
20	10.339

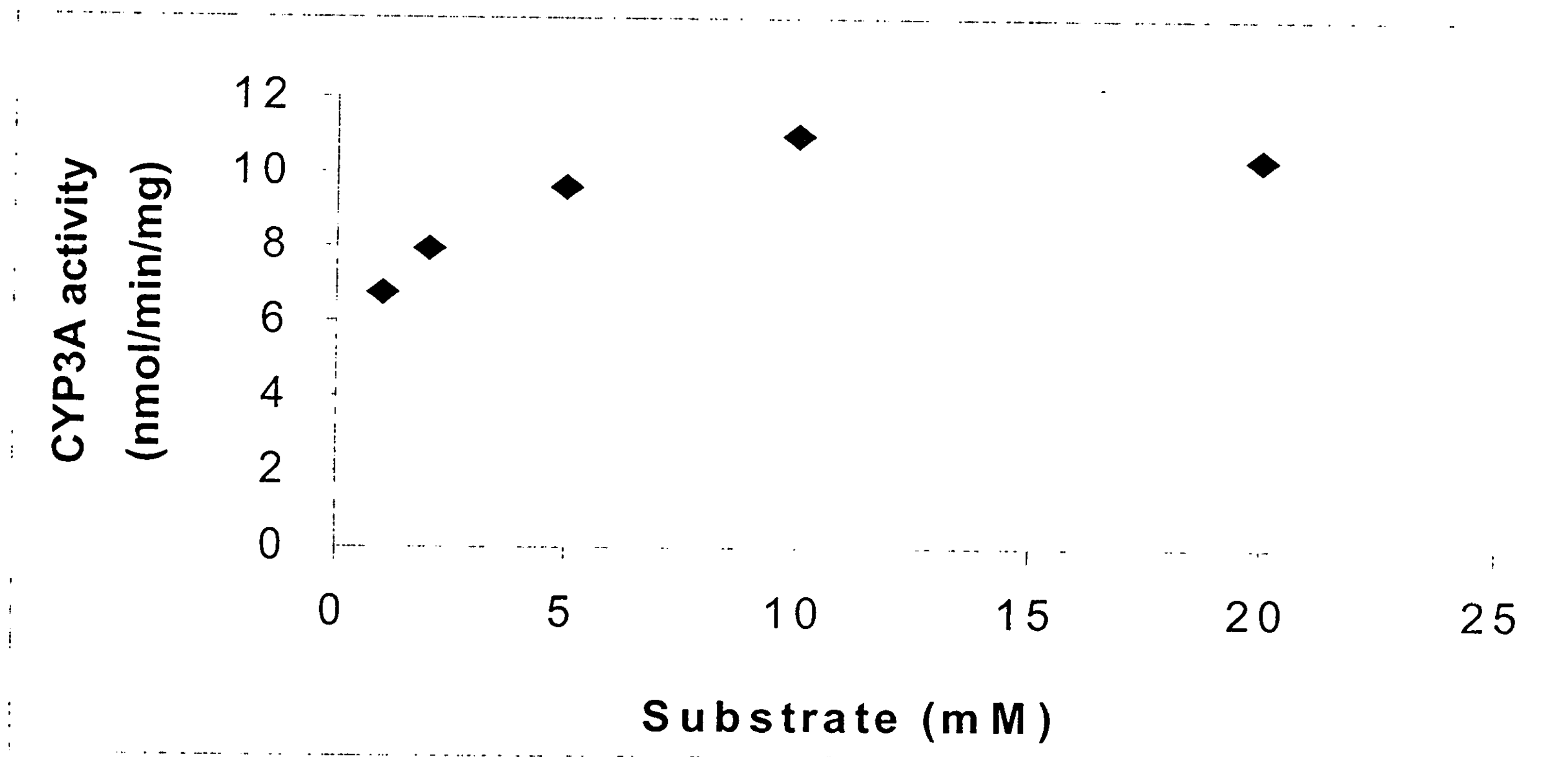


Figure 5.4 A plot of CYP3A activity at varying erythromycin (substrate) concentrations

5.6 Discussion

The formaldehyde calibrations proved to be reproducible with a coefficient of variation of less than 15 %. To compensate for the interday sensitivity of the spectrophotometer, a calibration was performed with every batch of enzyme assays.

The reaction conditions for the assay of rat microsomal CYP3A activity was successfully standardised. Optimum CYP3A activity was achieved at 0.8 mg/ml of microsomal protein with a substrate concentration of 5 mM and a reaction incubation time of 10 min. These conditions were used in the subsequent studies to measure microsomal CYP3A activity.

CHAPTER 6

DETERMINING THE OPTIMUM TIME OF INDUCTION OF RAT CYP3A

6.1 Introduction

Enzyme induction is defined as an increase in the amount or activity of an enzyme. Usually, the inducer drug stimulates the synthesis of a new enzyme, commonly by increasing transcription, but it can also occur by stabilising the protein at translational level. Although, enzyme induction is a complex process, the intricate mechanisms of which still remain unknown, it is characterised by a time lag after exposure to the inducer. As cited earlier in the review, it is known that nevirapine-induced hepatotoxicity commonly occurs from 2 – 4 weeks of treatment, the time at which enzyme induction is presumed to be optimum.

Since the aim of the study was to assess the role of CYP3A in nevirapine-induced hepatotoxicity, it was necessary to undertake tests at a time when the enzyme (CYP3A) activity was optimum. As such, in this chapter the use of dexamethasone and nevirapine itself, to induce rat CYP3A is described.

6.2 Reagents

Dexamethasone 21-phosphate, erythromycin, D-glucose-6-phosphate monosodium salt, β-Nicotineamide adenine dinucleotide phosphate sodium salt (NADP) and glucose-6-phosphate dehydrogenase were purchased from Sigma Chemical Co. (St. Louis, MO). Nevirapine as an oral suspension (50 mg/5 ml) Viramune® (Boehringer Ingelheim Pharmaceuticals, Inc., Ridgefield, CT, U.S.A) was purchased from a local pharmacy.

6.3 Method

6.3.1 Treatment with dexamethasone

Four groups of animals (Group A, B, C and D) of five rats each were treated with dexamethasone. Group A and B were treated with dexamethasone (30 mg/kg/day, intraperitoneally) for 3 and 5 days, respectively, while Group C and D were treated with dexamethasone (50 mg/kg/day, intraperitoneally) for 3 and 5 days, respectively.

The animals were sacrificed 24 hrs after the last dose. Under anaesthesia with diethyl ether, the abdomen was opened, abdominal contents gently pulled aside and blood (5 ml) was drawn from the abdominal vein in heparinised tubes and placed on ice immediately. The blood samples were centrifuged within 15 min and the supernatant (plasma) was sent for liver function tests. The liver was excised and washed in cold 1.15 % KCl after which a piece was kept in 10 % formalin for histology and the remainder was stored under liquid nitrogen until microsomes were extracted (described in chapter 4). Microsomes were used to measure the activity of CYP3A using the erythromycin demethylation test described in chapter 5.

6.3.2 Treatment with nevirapine

Four groups of animals (Group E, F, G and H) of five rats each were treated with nevirapine. Group E and F were treated with nevirapine (20 mg/kg/day, orally) for 3 and 5 days, respectively, while Group G and H were treated with nevirapine (40 mg/kg/day, orally) for 3 and 5 days, respectively. After the last dose, the animals were treated as described under the treatment with dexamethasone.

6.3.3 Analysis of results

The results of different groups were compared to determine the time and dose that achieved optimum CYP3A activity. The Mann-Whitney U Test was used for testing differences with a level of significance of 0.05 using GraphPad Instat, version 3.05, GraphPad Software, Inc.

6.4 Results

6.4.1 Treatment with dexamethasone

Table 6.1 shows CYP3A activity after treatment with dexamethasone, 30 mg/kg/day, while Table 6.2 shows that after treatment with 50 mg/kg/day (appendix B-1, B-2 and B-3). CYP3A activity was very low in the untreated animals (0.587 nmol/min/mg protein). At 30 mg/kg/day there was no significant difference ($P = 0.7537$) between CYP3A activity on day 3 (6.768 nmol/min/mg) and 5 (6.152 nmol/min/mg protein). Similarly there was no difference in CYP3A activity on the third (10.393 nmol/min/mg protein) and fifth (9.659 nmol/min/mg protein) day after treatment with 50 mg/kg/day ($P > 0.9999$). Of note, CYP3A activity was higher after 50 mg/kg/day than after 30 mg/kg/day on both days but this was not statistically significant ($P = 0.1508$), probably due to wide variations (Fig. 6.1). Therefore, dexamethasone 50 mg/kg/day for 3 days was selected as the dose and time at which optimum CYP3A activity was attained.

6.4.2 Treatment with nevirapine

Table 6.3 shows CYP3A activity after treatment with nevirapine, 20 mg/kg/day, while Table 6.4 shows that after treatment with 40 mg/kg/day (appendix B-4). Again, CYP3A activity was very low in the untreated animals (0.587 nmol/min/mg protein). At 20 mg/kg/day there was no significant difference ($P = 0.5476$) between CYP3A activity on day 3 (7.284 nmol/min/mg) and 5 (8.011 nmol/min/mg protein). Similarly there was no difference in CYP3A activity on the third (3.614 nmol/min/mg protein) and fifth (3.273 nmol/min/mg protein) day after treatment with 40 mg/kg/day, $P > 0.9999$. However, CYP3A activity was significantly lower after 40 mg/kg/day than after 20 mg/kg/day on both days (Fig. 6.2), $P = 0.0317$. Therefore, nevirapine 20 mg/kg/day for 3 days was selected as the dose and time at which optimum CYP3A activity was attained.

6.4.3 Comparison of dexamethasone and nevirapine treated rats

Although the optimum activity of CYP3A after treatment with dexamethasone (10.393 nmol/min/mg protein) was higher than nevirapine (7.284 nmol/min/mg protein), this was not statistically significant (P= 0.1508).

Table 6.1. CYP3A activity (nmol/min/mg protein) after 3 and 5 days of treatment with dexamethasone, 30 mg/kg/day.

Rat	Untreated	Group A CYP3A activity at 3 days	Group B CYP3A activity at 5 days
1	0.140	3.259	4.688
2	1.090	9.643	8.929
3	0.615	7.679	5.402
4	1.020	5.402	5.446
5	0.070	8.750	7.188
Average	**0.587**	**6.947**	**6.331**
± SD	**0.477**	**2.60**	**1.72**

P = 0.7537, not significant (Mann-Whitney U Test, 0.05 level of significance)

Table 6.2. CYP3A activity (nmol/min/mg protein) after 3 and 5 days treatment with dexamethasone, 50 mg/kg/day).

Rat	Untreated	Group C CYP3A activity at 3 days	Group D CYP3A activity at 5 days
1	0.140	9.893	9.268
2	1.090	16.277	9.402
3	0.615	7.929	8.643
4	1.020	7.170	11.321
5	0.070	10.696	-
Average	**0.587**	**10.393**	**9.658**
± SD	**0.477**	**3.586**	**1.157**

P > 0.9999, considered not significant (Mann-Whitney U Test, 0.05 level of significance)

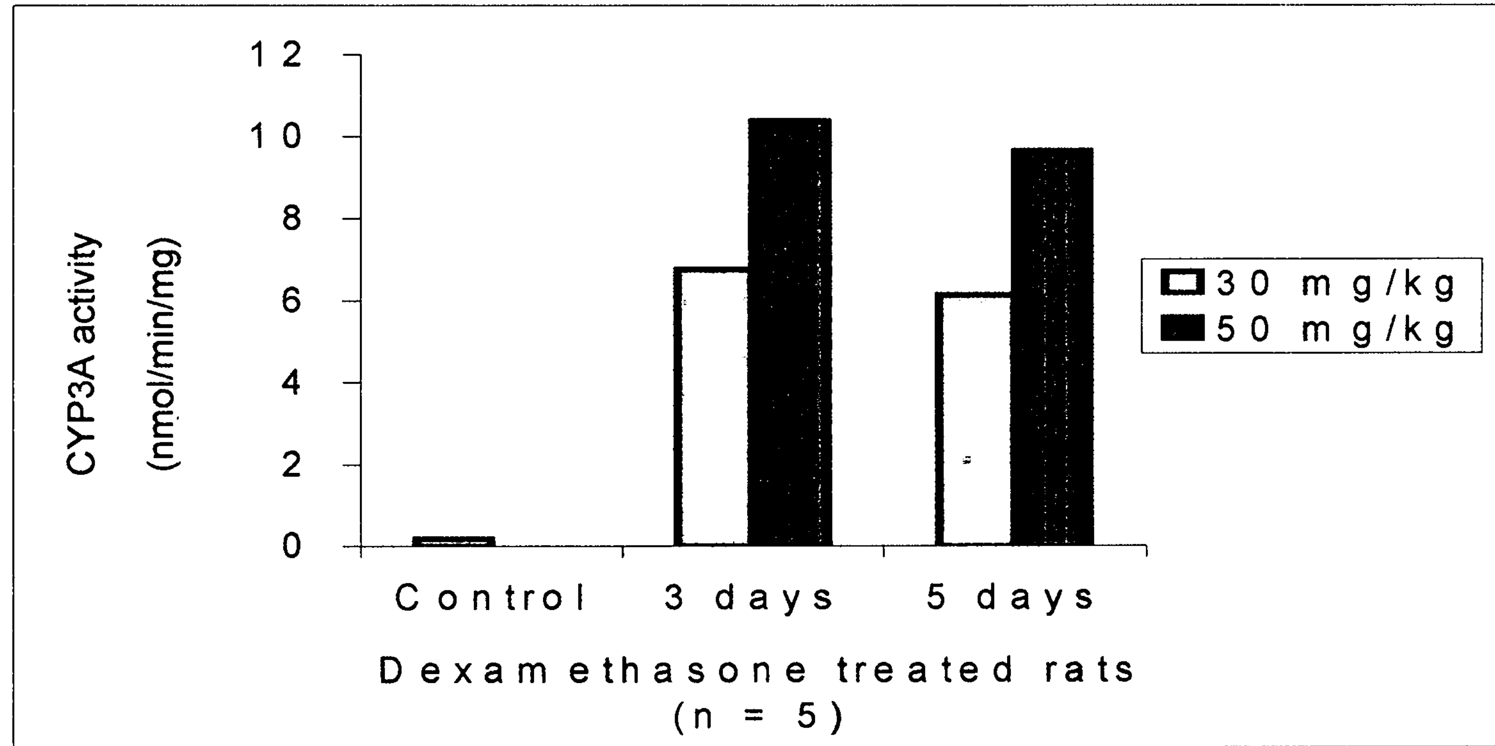


Figure 6.1 CYP3A activity after treatment with dexamethasone, 30 and 50 mg/kg/day.

Table 6.3. CYP3A activity (nmol/min/mg protein) after 3 and 5 days of nevirapine treatment, 20 mg/kg/day.

Rat	Untreated	Group E CYP3A activity at 3 days	Group F CYP3A activity at 5 days
1	0.140	9.830	7.386
2	1.090	3.920	10.511
3	0.615	5.739	8.182
4	1.020	10.057	7.841
5	0.070	6.875	6.136
Average	**0.587**	**7.284**	**8.011**
± SD	**0.477**	**2.648**	**1.598**

P = 0.5476, considered not significant (Mann-Whitney U Test, 0.05 level of significance)

Table 6.4. CYP3A activity (nmol/min/mg protein) after 3 and 5 days of nevirapine treatment, 40 mg/kg/day.

Rat	Untreated	Group G CYP3A activity at 3 days	Group H CYP3A activity at 5 days
1	0.140	5.626	3.977
2	1.090	2.670	1.307
3	0.615	4.205	5.170
4	1.020	2.727	2.955
5	0.070	2.841	2.955
Average	**0.587**	**3.614**	**3.273**
± SD	**0.477**	**1.291**	**1.429**

P > 0.9999, not significant (Mann-Whitney U Test, 0.05 level of significance)

SD = standard of deviation

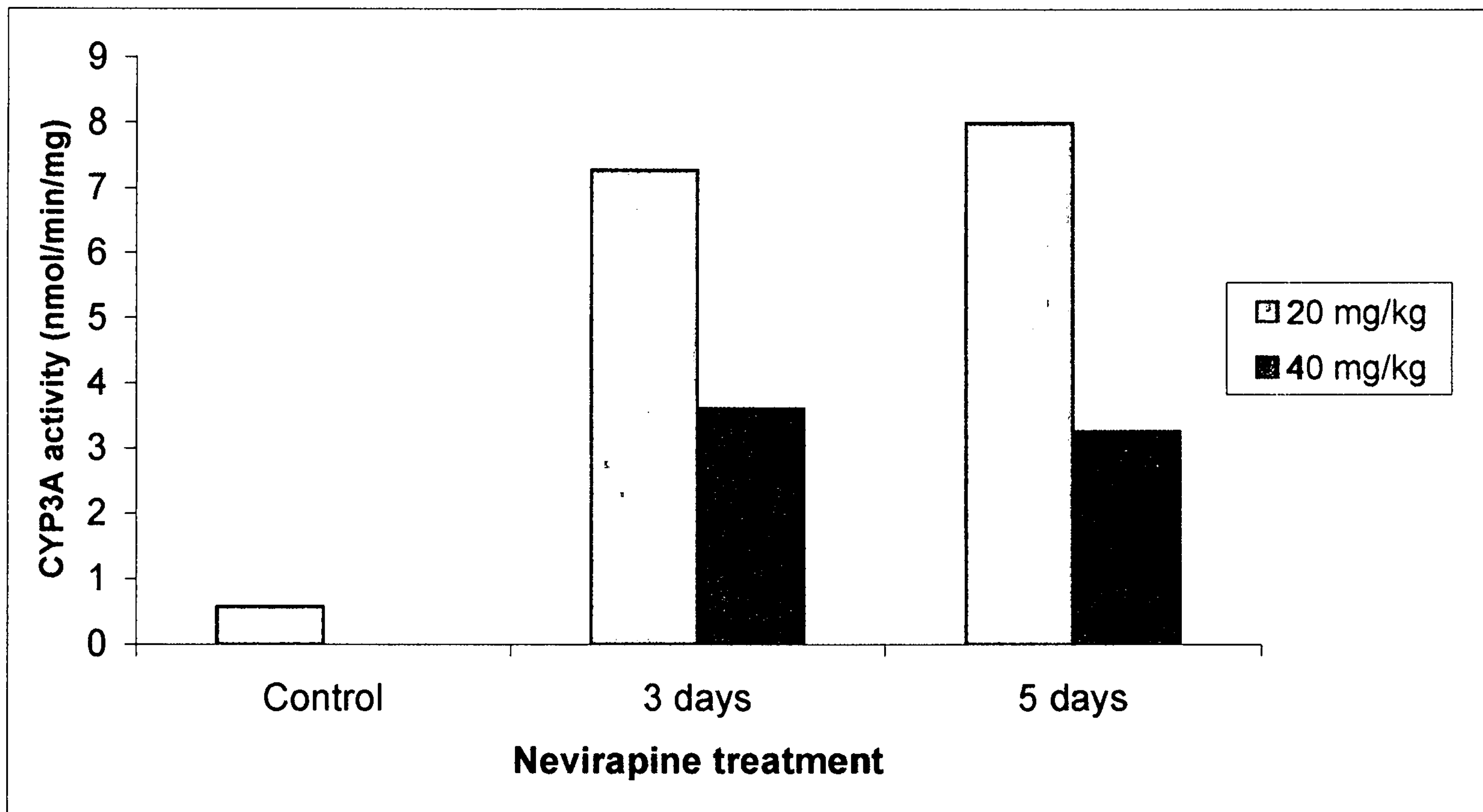


Figure 6.2 CYP3A activity after treatment with nevirapine, 20 and 40 mg/kg/day.

6.5 Discussion

Both dexamethasone and nevirapine treatment lead to an increase in CYP3A activity when compared to untreated rats, confirming that both these drugs are inducers of CYP3A. Administering dexamethasone 50 mg/kg/day for 3 days attained optimum enzyme activity, whereas nevirapine treated rats revealed optimum enzyme activity at a dose of 20 mg/kg/day for 3 days.

Interestingly, nevirapine 40 mg/kg/day was associated with a decreased activity of CYP3A. This was probably because it was a very high dose of nevirapine that could cause liver damage or enzyme saturation. Alternatively, it could have also been due to the fact that nevirapine mediated CYP3A induction is not dose dependent.

In conclusion, optimum dose of CYP3A induction has been achieved for both dexamethasone and nevirapine, and this was used in the subsequent experiments to determine the role of CYP3A in nevirapine-induced hepatotoxicity.

CHAPTER 7

CYP3A QUANTITATION BY SDS-PAGE AND WESTERN BLOTTING

7.1 Introduction

As mentioned earlier, enzyme induction can lead to increased enzyme quantity or activity. In the previous chapter, increased activity of CYP3A was observed on pre-treatment with dexamethasone and nevirapine. However, it is not known whether this increase in enzyme activity is due to increase in enzyme quantity or improved function of the enzyme not related to change in enzyme quantity. Therefore, in this section, the quantitation of CYP3A activity by SDS-PAGE and Western blot techniques in the animals treated with dexamethasone and nevirapine is described.

Of note, it is important to inform the reader that although this method was successfully set up, the detection of CYP3A was not satisfactory due to a wrong antibody that was supplied.

7.2 Reagents

Sigma Chemical Co. (St Louis, MO USA) supplied TrizmaBase (Tris), sodium dodecyl sulphate (SDS), acrylamide/bis-acrylamide 40 % solution, ammonium persulfate (AMPS), N, N, N', N' - tetramethylenediamine (TEMED), glycerol, brilliant blue G-colloidal concentrate, bromophenol blue, ponceau S solution 0.1% (w/v) and blotting paper. Hybond PVDF membrane, ECL protein molecular weight marker and the ECL plus detection reagents were purchased from Amersham Pharmacia Biotech Inc. (Sweden). PanVera supplied the primary antibody: Anti-P450 3A4/5 Monoclonal mouse IgM, 31.8 mg/ml. The ECL Anti-mouse IgM, horseradish peroxidase linked whole antibody was used as the secondary antibody and was purchased from Amersham Biosciences, (U.K., Buckinghamshire). Protea, instant fat free milk powder was used for blocking. USB (Cleveland, Ohio) supplied the glycine and BDH laboratory supplies (England, U.K.) supplied the sodium dihydrogen orthophosphate dihydrate and the di-sodium hydrogen orthophosphate. Tween-20 was purchased from Merck (Darmstadt, Germany) and the methanol was obtained from Labscan (Dublin, Ireland) and was of analytical grade.

7.3 Apparatus

A Mini PROTEAN II cell component and accessories (BIO-RAD) were used to perform the SDS-PAGE using a Power PAC 300 (BIO-RAD) as the electric current source. The Mini Trans-Blot electrophoretic transfer cell component (BIO-RAD) was used to transfer the proteins from the gel onto the membrane. An orbital shaker S03 (Stuart Scientific, UK) was used for the membrane washings and antibody incubations.

7.4 Methods

7.4.1 Preparation of buffers

7.4.1.1 12 % Separating gel

A 12 % Separating gel was prepared by mixing the following chemicals, 3.75 ml of 1 M Tris-HCl, pH 8.8, 3 ml of 40 % acrylamide/bis, 100 μl of 10 % SDS and 3.1 ml water. A stock solution of 100 ml was prepared of which, for one experiment 10 ml was used to prepare a pair of gels. Gel setting was affected just prior to gel pouring by addition of 50 μl of 10 % AMPS and 5 μl of TEMED to 10 ml of the stock solution.

7.4.1.2 4 % Stacking gel

A 4 % Stacking Gel was prepared by mixing 1.25 ml of 1 M Tris-HCl, pH 6.8, 1 ml of 40 % acrylamide/bis and 100 μl of 10 % SDS and 7.6 ml water. A stock solution of 100 ml was prepared of which, for one experiment 8 ml was used to prepare a pair of gels. Gel setting was affected just prior to gel pouring by addition of 50 μl of 10 % AMPS and 10 μl of TEMED to 8 ml stock solution.

7.4.1.3 Sampling Buffer

The sampling buffer was prepared by adding 5 ml of 1 M Tris-HCl (pH 6.8), 8 ml glycerol, 16 ml 10 % SDS, 4 ml of 2-mercaptoethanol, 4 ml of 1 % bromophenol Blue and 43 ml water.

7.4.1.4 Running Buffer (TGS Buffer)

The 10X TGS Buffer (pH 8.3) consisted of 30.3 g Tris-HCl, 144.1 g glycine, 100 ml 10 % SDS and 800 ml distilled water. One hundred millilitres of this buffer was diluted

to 1 litre, to obtain final concentrations of 0.1 % SDS, 25 mM Tris and 192 mM glycine.

7.4.1.5 Protein Transfer Buffer

The Protein Transfer Buffer was prepared in final concentrations of 25 mM Tris and 192 mM glycine in 20 % methanol.

7.4.1.6 PBS and PBS-T

Phosphate buffered saline (PBS) pH 7.5 consisted of sodium chloride, sodium dihydrogen orthophosphate di-hydrate and di-sodium orthophosphate anhydrous in final concentrations of 100 mM, 20 mM and 80 mM respectively. This buffer was used to prepare the blocking buffer (PBS-T) that was comprised of 0.1 % Tween 20 in PBS.

7.4.1.7 ECL detection reagents

The ECL detection kit consisted of solution A and B. Solution A was an ECL substrate solution containing a Tris buffer and Solution B was comprised of a stock Acridan solution of dioxane and ethanol.

7.4.2 Sample preparation

Known volumes of microsomal samples whose protein concentrations had been determined as previously described were diluted with sampling buffer, after which they were heated for 3 min on boiling water and then placed on ice. The molecular weight marker was treated in the same way. The samples were then centrifuged for 20 seconds, after which they were ready for loading on the gel.

7.4.3 SDS PAGE and CYP3A detection by Western blot

7.4.3.1 Gel preparation

Two glass plates were first cleaned with ethanol after which they were assembled with spacers on either side placed between them to create a container in which the gel was prepared. The separating gel (prepared as in 7.4.1.1) was poured between the glass plates up to a mark, 2 cm from the top. Isopropanol was overlaid to ensure uniform setting of the gel. The gel was then left to set for approximately 20 min after which isopropanol was removed by rinsing with water. The excess water drops on

the glass plates were blotted dry. The stacking gel (prepared as in 7.4.1.2) was poured on top of the separating gel to fill up the container. The comb was then inserted to create wells in which the samples were to be loaded. The gel was left to set for 20 min.

7.4.3.2 Electrophoresis

The gel, held between the plates, was transferred into the electrophoretic apparatus containing TGS buffer in which it was pre-conditioned by applying a 100 V electric current for about 45 min. The wells were rinsed with TGS Buffer and the samples were loaded carefully. The gel was run for 2 hours at 100 V (until the loading front reached the bottom of the gel) after which the glass plates were separated and the stacking gel was removed. The left-hand upper corner of the separating gel was cut for orientation purposes and the gel was prepared for electro-transfer.

7.4.3.3 Electro-transfer

Briefly, the left-hand upper corner of the PVDF membrane was cut to correspond to the cut on the gel after which it was pre-wet with methanol for 10 seconds and then washed with water. Thereafter the PVDF membrane was placed between two blotting papers and these were inserted between two fibre pads. The unit was immersed in a protein transfer buffer for 15 min at 4 °C (in the refrigerator) to equilibrate, after which it was opened and the gel was placed on the membrane in the appropriate orientation. The fibre pads with the gel were locked into a cassette and this was referred to as the gel sandwich (see literature review). The gel sandwich was inserted into the tank containing protein transfer buffer to which an electric power of 100 V was applied for 1 hour to transfer proteins to the membrane (electro-transfer). The membrane was removed and stained with 2 ml (0.1 %) Ponceau S solution for 2 min to determine whether the proteins were transferred onto the membrane.

7.4.3.4 Blotting

The membrane was washed with water after which it was incubated for one hour in 5 % (w/v) dried non-fat milk dissolved in PBS-T, supposedly to block non-specific sites. The membrane was then washed with plain PBS-T buffer (2 x 5 minutes) followed by incubation in the same buffer containing the primary antibody (1:2500) for one hour. It was then washed three times in PBS-T buffer (2 x 5 minutes and 1 x 15 minutes)

after which it was incubated in the secondary antibody (1:2000) for 1 hour. There after, the membrane was washed three times (2 x 5 minutes and 1 x 15 minutes) with PBS-T buffer.

7.4.3.5 ECL detection

CYP3A was detected using the ECL Plus detection kit according to manufacturers instructions (see literature review). Briefly, the ECL detection reagents were allowed to reach room temperature. Solutions A and B were mixed in a ratio of 40:1 (2000 μl of solution A and 50 μl of solution B was sufficient for one blot). After draining off the excess wash buffer, the membrane was placed, with protein side up, on a clean piece of Glad-wrap. The detection mixture was applied to the membrane and incubated in the dark for 5 min at room temperature. After draining off the detection mixture, the membrane was placed on a clean wrap, but this time with the protein side down, and the wrap was closed. The protein side of the membrane was exposed in the dark to a Hyperfilm ECL (Amersham) for approximately 15 seconds and the film was developed using Agfa developer and fixer. CYP3A was visible as black bands between 40 – 60 kDa.

7.4.4 Optimisation of experimental conditions

The following steps were performed to optimise the Western blot conditions:

7.4.4.1 Amount of sample to load

Firstly, protein samples were loaded on the gel in a concentration range of 0.5, 1, 2.5, 5, 10 and 15 μg. The aforementioned SDS-Page and Western blot conditions were performed with the inclusion of a molecular marker in the first well. ***Result:*** There was overloading of protein on the gel (Fig. 7.1), a lower concentration range was therefore used, 0.1, 0.25, 0.5, 1 and 2.5 μg (Fig. 7.2). Further optimisation was required as too much background was observed on the blot.

7.4.4.2 Primary antibody

The same serial dilution of proteins with the inclusion of a molecular marker was run using a more diluted primary antibody, 1:5000. ***Result:*** The background was not improved and the bands were far less detectable (Fig. 7.3).

7.4.4.3 Secondary antibody

A serial dilution of 0.75, 1, 1.5 and 2 μg was run on a gel. Washes were increased to 2 x 3 min, 1 x 10 min and 1 x 15 min, after the antibody incubations. Secondary antibody concentrations of 1:2000 and 1:3000 were compared. ***Result:*** There was a slight improvement in the background and the secondary antibody concentration of 1:2000 (Fig. 7.4). The dilution of 1:3000 showed high background, non-specific binding and the protein bands were of a poor quality.

7.4.4.4 Run time

Electro-transfer was done for 2 hours at 100 V as opposed to the original 1 hour, but because of the heat, the cooling unit was replaced after one hour. ***Result:*** This resulted in satisfactory protein detection, with the elimination of background (Fig. 7.5).

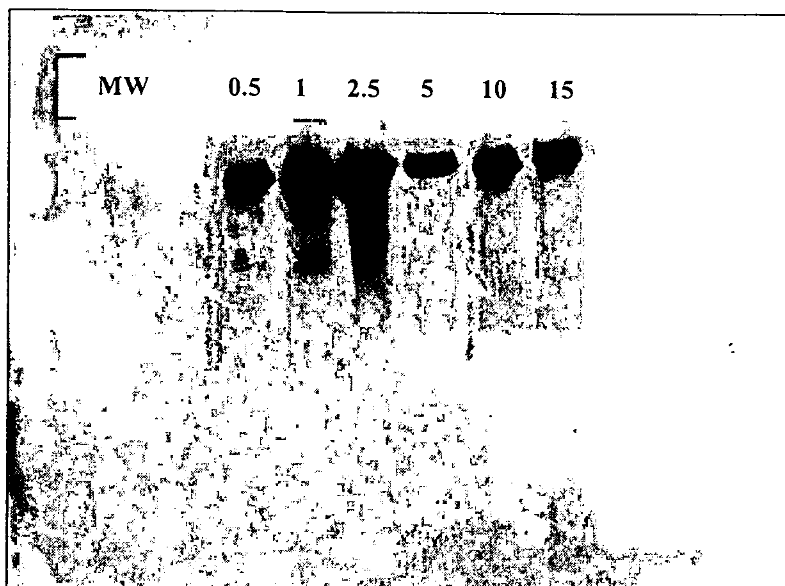


Figure 7.1 Western blot of CYP3A indicating signals of the different amounts of microsomal protein loaded (0.5, 1, 2.5, 5, 10 and 15 μg), after incubation with primary antibody dilution of 1:2500.

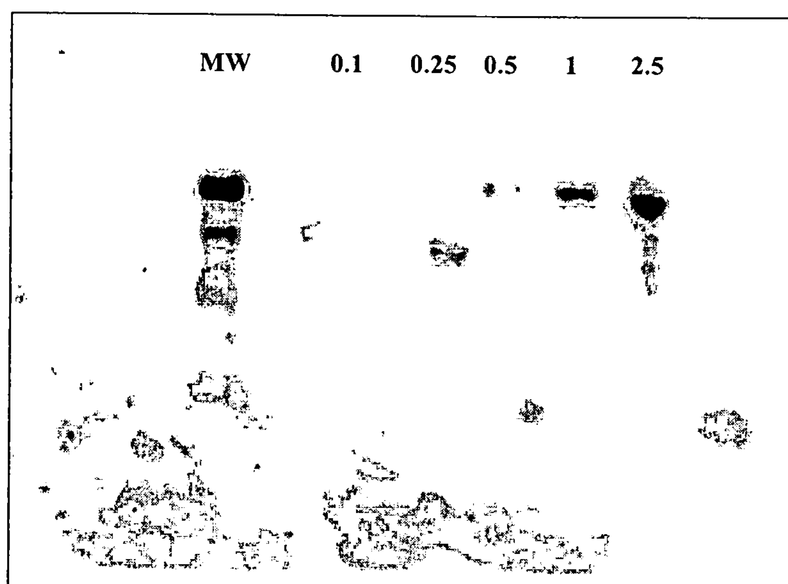


Figure 7.2 Western blot of CYP3A indicating signals of the different amounts of microsomal protein loaded (0.1, 0.25, 0.5, 1 and 2.5 μg) after incubation with primary antibody dilution of 1:2500.

Figure 7.3 Western blot of CYP3A supposed to indicate signals of the different amounts of microsomal protein loaded (0.1, 0.25, 0.5, 1 and 2.5 μg) after incubation with primary antibody dilution of 1:5000.

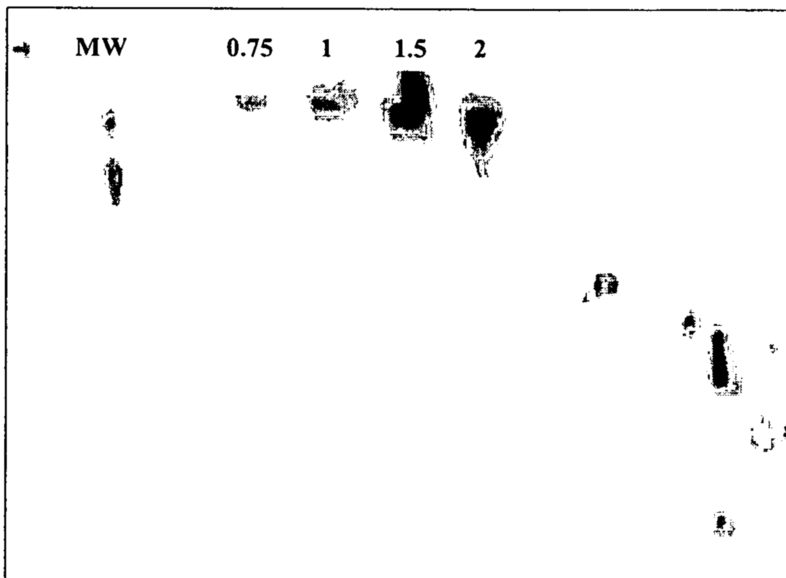


Figure 7.4 Western blot of CYP3A supposed to indicate signals of the different amounts of microsomal protein loaded (0.75, 1, 1.5 and 2. μg). Secondary antibody dilution was 1:2000.

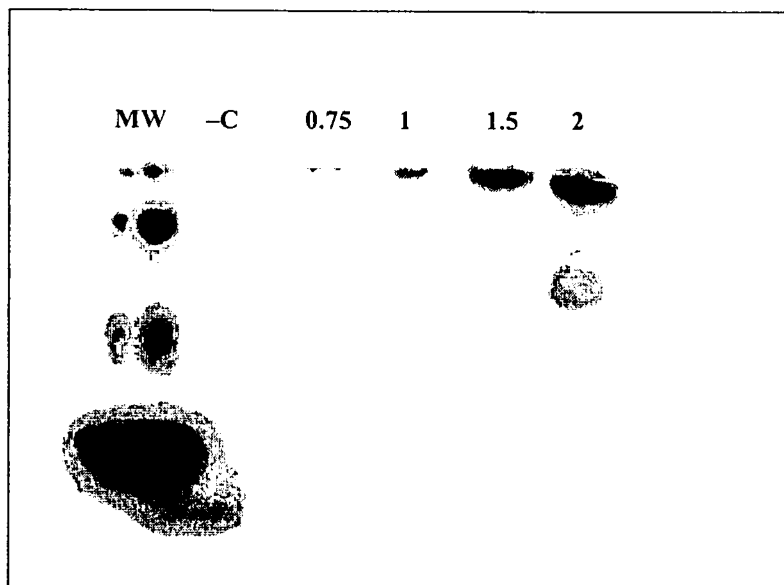


Figure 7.5. Western blot of CYP3A indicating signals of the different amounts of microsomal protein loaded (0.75, 1, 1.5 and 2. μg). Electro-transfer was for 2 hours at 100 V.

7.4.5 The final adjustments to the procedure for CYP3A detection

- Sample load: 1 μg was used as determined from the blot depicted in Figure 7.5.
- Primary antibody: dilution of 1:2500, incubated overnight at 4 °C.
- Secondary antibody: dilution of 1:2000, incubated for 1 hour at room temperature.
- Washing with PBS-T: after the primary and secondary antibody incubations the washing times were increased to 2 x 3 min, 1 x 10 min and 1 x 15 min.
- Electro-transfer: 2 hours at 100 V and cooling unit was replaced after 1 hour.

This procedure was used in the subsequent experiments to determine the effect of dexamethasone and nevirapine on the quantity of CYP3A.

7.5 Effect of dexamethasone and nevirapine on CYP3A

Microsomal samples from untreated rats and from those treated with dexamethasone or nevirapine were prepared as described in sample preparation. The gel was prepared as described earlier and microsomal samples (1 μg) were loaded and the procedure of electrophoresis, electro-transfer and CYP3A detection by western blot were performed as described above.

Quantitation of CYP3A was done on the following groups of rats: untreated animals (n = 5), rats treated with 20 mg/kg/day of nevirapine for 3 and 5 days, (n = 5) and rats treated with 30 mg/kg/day dexamethasone for 3 and 5 days, (n = 5).

7.6 Results

Figure 7.6 is supposed to illustrate amount of CYP3A in untreated rats. Unfortunately, it was hard to interpret as it appears there was poor transfer of the protein. However, this could also be due to lack of expression of CYP3A in untreated rats as observed earlier (previous chapter) where there was minimal or no CYP3A activity.

Figure 7.7 (A and B) shows the blot of CYP3A in samples of rats that were treated with nevirapine for 3 and 5 days, respectively. On both occasions, there was a relative increase in the amount of CYP3A in rat no's. 1, 2, 3 and 5, indicating significant induction of CYP3A by nevirapine. Even here the blots were not satisfactory as evidenced by poor detection of the molecular weight marker.

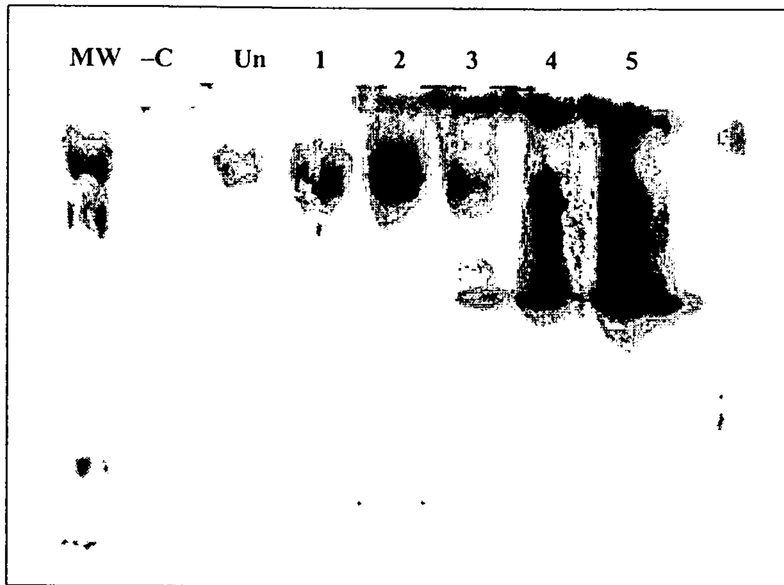
Figure 7.8 (A and B) shows the blot of CYP3A in samples of rats that were treated with dexamethasone for 3 and 5 days, respectively. Whereas no enzyme was detected on day 3, there appeared to be a moderate increase in the amount of CYP3A on day 5 in rat no's. 1, 2, 3 and 5, indicating significant induction of CYP3A by nevirapine. Despite several attempts, there was no improvement, hence, these results were not acceptable.

7.6.1 Trouble shooting

It was then noted that the primary antibody, according to manufacturer's instruction was not suitable for immunoblotting. It was also noted that the more the antibody was re-used, the less background was observed. This information was not available in the catalogue or from the supplier at the time of purchasing.

Figure 7.6 A blot of CYP3A in microsomes from untreated rats.

A



B

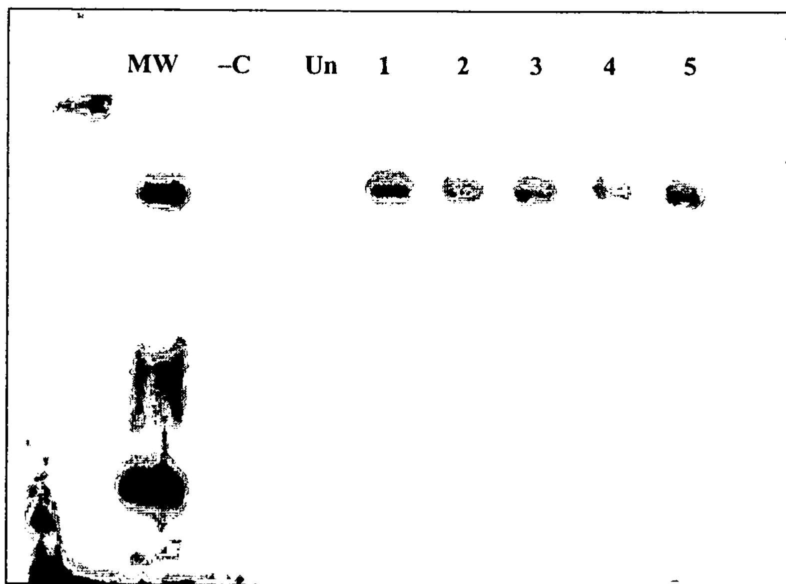
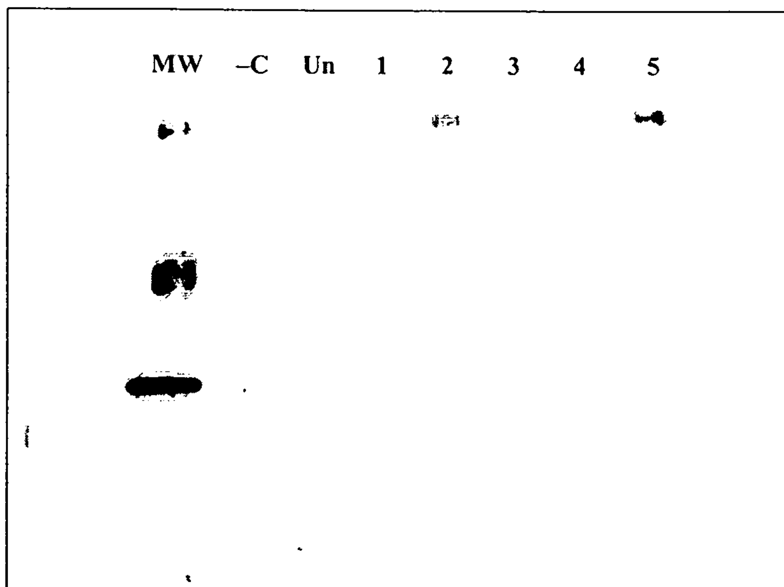


Figure 7.7 A blot of CYP3A in microsomes from rats treated with nevirapine for 3 days (A) and 5 days (B).

A



B

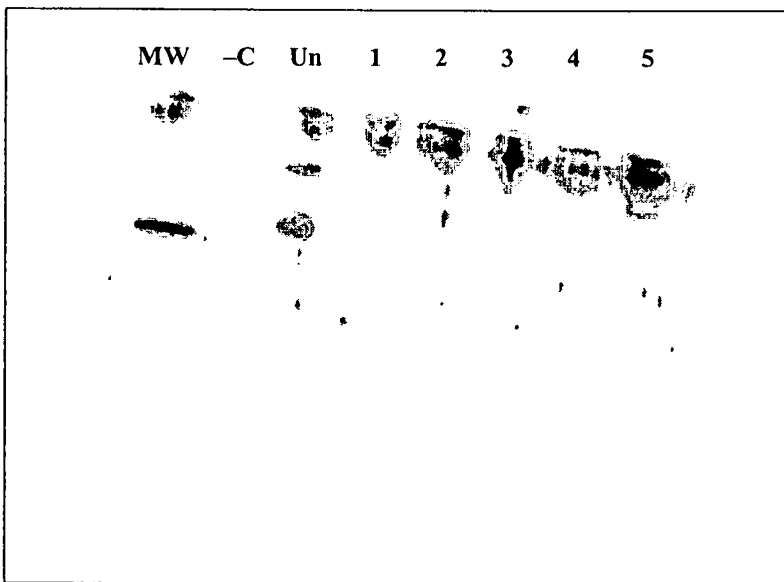


Figure 7.8 A blot of CYP3A in microsomes from rats treated with dexamethasone for 3 days (A) and 5 days (B).

7.7 Discussion

The conditions of SDS-PAGE and western blotting were optimised, however these conditions did not seem fit for the application thereof. High background, non-specific binding as well as inconsistent results was obtained. Since the buffers were freshly prepared, they were not responsible for the poor detection of CYP3A. The detection of CYP3A especially in the groups treated with dexamethasone was very poor. These groups however showed high CYP3A activity as described in chapter 6, indicating that these results were inconclusive.

It was however noted that the more the antibody was re-used, the clearer the background was, this was possibly due to the antibody not being suitable for immunoblotting studies (according to manufacturer's instructions) therefore resulting in inconsistent blots.

This method was considered inconclusive and further optimisation or the use of a different primary antibody was required. Since the results of CYP3A activity (chapter 6) showed that CYP3A was induced by treatment with nevirapine and dexamethasone, it was decided to continue to the next phase of testing the role CYP3A in nevirapine induced hepatotoxicity. The hope was that this would determine whether it would be necessary to spend more money and secure an appropriate antibody for the experiment.

CHAPTER 8

THE ROLE OF CYP3A INHIBITORS IN THE PREVENTION OF NEVIRAPINE INDUCED HEPATOXICITY

8.1 Introduction

In this chapter, investigations on the role of CYP3A in nevirapine-induced hepatotoxicity are explained. First, is a study on the acute hepatotoxicity of nevirapine in normal rats, followed by a similar study but in rats pre-treated with inducers of CYP3A, i.e., nevirapine or dexamethasone. Since it was demonstrated in the previous chapters that treatment with dexamethasone or nevirapine lead to induction of CYP3A, the role of CYP3A in nevirapine-induced hepatotoxicity was determined by pre-treatment with a CYP3A inhibitor.

8.2 Reagents and apparatus

Nevirapine (Viramune®; Boehringer Ingelheim Pharmaceuticals, Inc., Ridgefield, CT, U.S.A) syrup and tablets were obtained from a local pharmacy. Sigma Chemical Co. (St. Louis, MO) supplied the dexamethasone 21-phosphate disodium salt and the ketoconazole. Feeding needles (16 G-3", curved 3.00 mm ball) for oral gavage, were purchased from popper & sons, Inc., (New York, U.S.A) and a dissection kit obtained from Lasec (Bloemfontein, South Africa) was used to perform the surgical procedure. Heparin coated glass tubes were used to collect blood and an eppendorf centrifuge (5810 R) was used to obtain plasma from the collected blood.

8.3 Methods

8.3.1 Animal experiment

Male Sprague-Dawley rats weighing 300 – 350 g were used and the standard surgical procedure was performed as described in chapter 4. Blood was collected, centrifuged and plasma was sent for liver function tests. A piece of the liver was stored in 10 % formalin and was sent for analysis, blindly by an independent pathologist, Dr WS Botha, VetPath (Pretoria, South Africa).

8.3.2 Statistical analysis

The Mann-Whitney U Test with level of significance at $P < 0.05$ was used to compare the liver function tests of the different groups (Instat GraphPad Software, Inc., version 3.05).

8.3.3 PART I: TEST FOR NEVIRAPINE INDUCED HEPATOTOXICITY IN NORMAL RATS

In this respect, normal rats, refers to animals that were not pre-treated with dexamethasone or nevirapine.

8.3.3.1 Experiment

Animals were divided into three groups of five rats each (Group A, B and C). Group A was not treated with anything and was used as the control. Group B and C were treated with nevirapine (1340 mg/kg in two divided doses, 6 hours apart) by oral gavage, but group C was treated with ketoconazole (10 mg/kg, i.p.) an hour before the nevirapine dose was administered. The animals were sacrificed 24 hrs after the initial nevirapine dose. The standard surgical and laboratory procedures were performed as previously described and plasma was sent for liver function tests.

8.3.3.2 Results

Table 8.1 shows the liver function tests of the control group (Group A) and those treated with 'nevirapine only' (Group B). There was no significant difference in the ALP or ALT levels, however, there was a significant difference in the AST values indicating mild hepatotoxicity.

Table 8.1 Liver function tests of rats in the control group (Group A) and the group treated with 'nevirapine only' (Group B).

	Control Group A	Nevirapine only Group B	P value
	Mean ± SD (n = 5)	Mean ± SD (n = 5)	
ALP	174.2 ± 25.004	205.40 ± 26.894	0.0952
AST	101.80 ± 18.431	211.40 ± 53.435	0.0079*
ALT	108.00 ± 36.159	94.20 ± 23.091	0.3095

(Mann-Whitney U Test, 0.05 level of significance)

In Table 8.2 the liver function tests of rats treated with 'nevirapine only' (Group B) and those treated with 'ketoconazole plus nevirapine' (Group C) were compared. There was no significant difference between the liver enzyme levels ($p > 0.05$), in the two groups, implying that ketoconazole did not prevent the rise in AST or occurrence of mild hepatotoxicity (appendix C)

Table 8.2 Liver function tests of rats treated with 'nevirapine only' and those treated with the 'ketoconazole plus nevirapine'.

	Nevirapine Group B	Ketoconazole and nevirapine Group C	P value
	Mean ± SD (n = 5)	Mean ± SD (n = 5)	
ALP	205.40 ± 26.894	187.80 ± 18.267	0.2222
AST	211.40 ± 53.435	195.80 ± 62.078	0.3095
ALT	94.20 ± 23.091	78.80 ± 14.184	0.2222

(Mann-Whitney U Test, 0.05 level of significance)

Figure 8.1 is a diagrammatic illustration of the liver function tests of the three groups. From this figure it is clear that these three groups were relatively similar, except for the raised AST in rats treated with nevirapine, group B and C, signifying mild hepatotoxicity.

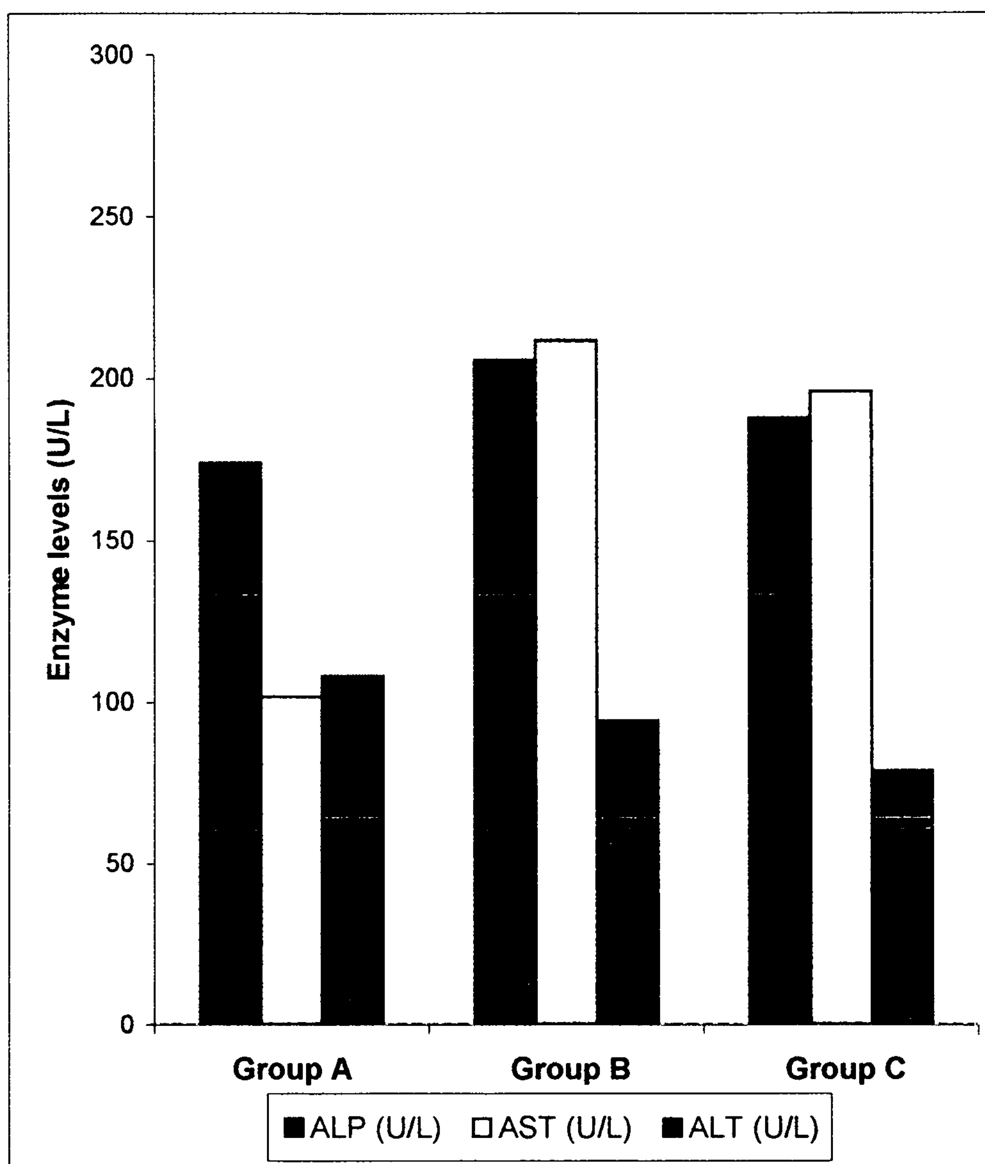


Figure 8.1 Liver function tests for rats in the control group (Group A), the group treated with 'nevirapine only' (Group B) and the group treated with the 'inhibitor and nevirapine' (Group C).

8.3.3.3 Comment

Although the level of AST was elevated in the group treated with 'nevirapine only' compared to the control group, there was no significant difference in the ALP and ALT values. Because ALT is the enzyme most specific for liver injury, it was concluded that severe hepatotoxicity was not attained in normal rats treated with nevirapine alone.

Furthermore, the lack of hepatotoxicity in rats treated with such a high dose of nevirapine, and its similarity to the 'ketoconazole plus nevirapine' group, was probably because CYP3A was not highly active in normal animals. This was confirmed by observations in the previous chapter, where it was demonstrated that control rats (normal rats) had minimal CYP3A activity. In such a situation, the use of a CYP3A inhibitor (ketoconazole) would not be helpful because there was no enzyme to inhibit. Due to the lack of nevirapine-induced hepatotoxicity in normal rats, it was decided to apply this experiment in rats pre-treated with a CYP3A enzyme inducer (dexamethasone or nevirapine), as this would result in a more active enzyme (explained in Part II and III of this chapter).

8.3.4 PART II: PRE-TREATMENT WITH NEVIRAPINE

8.3.4.1 Experiment

Again, the animals were divided into four groups (Group D, E, F and G) of five rats each. All groups were pre-treated orally with nevirapine (20 mg/kg/day) for 3 days. On the fourth day, Group D was treated with water (control group) while Group E was treated with 'ketoconazole only' (20 mg/kg, i.p.). Group F and G were administered nevirapine (1340 mg/kg, orally in two divided doses, 6 hrs apart), but Group G was treated with ketoconazole (20 mg/kg, i.p.) an hour before nevirapine was administered. Unlike in Part I, where 10 mg/kg ketoconazole was used to inhibit CYP3A in normal rats, a higher dose of ketoconazole (20 mg/kg) was used here because it was thought that a higher amount of inhibitor was required to overcome the increased activity of CYP3A induced by nevirapine pre-treatment. The animals were sacrificed 24 hrs after the initial dose of nevirapine was administered. The standard surgical and laboratory procedures were performed and plasma was sent for liver function tests, selected liver sections were sent for histology.

8.3.4.2 Results

Table 8.3 shows the liver function tests of the control group (Group D) and the 'ketoconazole only' treated group (Group E). There was no difference between the liver enzymes (AST and ALT) in the control group and the 'ketoconazole only' treated group (appendix D).

Table 8.3 Liver function tests of rats in the control group (Group D) and those in the 'ketoconazole only' treated group (Group E).

	Control Group D	Ketoconazole only Group E	P value
	Mean ± SD (n = 5)	Mean ± SD (n = 5)	
ALP	248.60 ± 46.01	151.40 ± 17.64	0.0079*
AST	119.80 ± 21.99	167.00 ± 51.45	0.0952
ALT	93.80 ± 31.67	102.80 ± 19.37	0.8413

(Mann-Whitney U Test, 0.05 level of significance)

Table 8.4 shows the liver function tests of the control group (Group D) and the 'nevirapine only' treated group (Group F). There was no difference between the liver enzymes (AST and ALT) in the control group and the 'nevirapine only' treated group (Group F). The high levels of ALP in the control group when compared to the 'ketoconazole only' and 'nevirapine only' treated groups could not be explained. Nevertheless ALP is not indicative of hepatocyte toxicity.

Table 8.4 Liver function tests of rats in the control group (Group D) and those in the 'nevirapine only' treated group (Group F).

	Control Group D	Nevirapine only Group F	P value
	Mean ± SD (n = 5)	Mean ± SD (n = 5)	
ALP	248.60 ± 46.01	167.60 ± 37.11	0.0317*
AST	119.80 ± 21.99	150.20 ± 19.11	0.0952
ALT	93.80 ± 31.67	67.20 ± 8.64	0.0556

(Mann-Whitney U Test, 0.05 level of significance)

The liver enzymes (ALP and AST) of the rats treated with the 'ketoconazole plus nevirapine' (Group G) were not significantly different from those treated with 'nevirapine only' (Group F). However there was a significant elevation in ALT levels of the 'ketoconazole plus nevirapine' group (Table 8.5), indicating that the use of ketoconazole may have had a synergistic toxic effect when combined with the high dose of nevirapine or that the dose of ketoconazole was too high when combined with nevirapine. However, according to these liver function tests, hepatotoxicity was not attained in the 'nevirapine only' treated group and therefore the use of ketoconazole as a preventative strategy could not be demonstrated here.

Table 8.5 Liver function tests of rats in the 'nevirapine only' treated group (Group E) and the 'ketoconazole plus nevirapine' treated group (Group F).

	Nevirapine only Group E	**Ketoconazole plus nevirapine Group F**	**P value**
	Mean ± SD (n = 5)	Mean ± SD (n = 5)	
ALP	167.60 ± 37.11	211.40 ± 30.85	0.0952
AST	150.20 ± 19.11	258.80 ± 192.86	0.3095
ALT	67.20 ± 8.64	130.00 ± 27.58	0.0079*

(Mann-Whitney U Test, 0.05 level of significance)

Figure 8.2 illustrates that there was no difference between the liver enzymes of the control group and the 'nevirapine only' treated group.

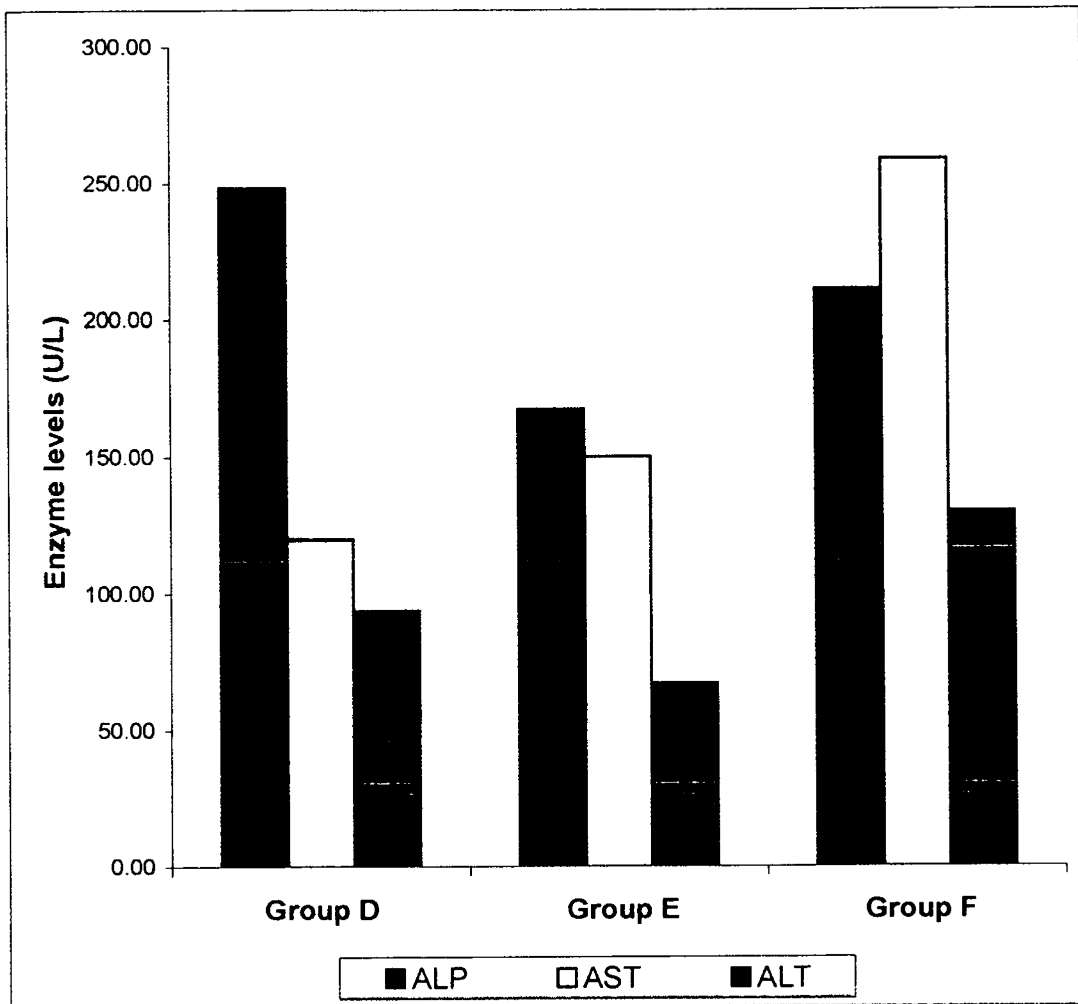


Figure 8.2 Liver function tests of rats in the control group (Group D), the 'nevirapine only' treated group (Group E) and the 'ketoconazole plus nevirapine' group (Group F).

8.3.4.3 Histopathological findings of rats pre-treated with nevirapine.

The following groups were selected for histology: Control group (Group D) and the 'nevirapine only' (Group E). Below are the histopathology reports, as reported by the pathologist.

Control group. "In this group all except one liver section shows mild to moderate cellular hypertrophy while mild hepatocellular degeneration and cell swelling could be confirmed in two rats" (Fig. 8.3).

'Nevirapine only' treated group: "In the liver sections of all rats in this group mild to severe hypertrophic changes could be detected while three of the rats show mild to moderate apoptosis" (Fig. 8.4).

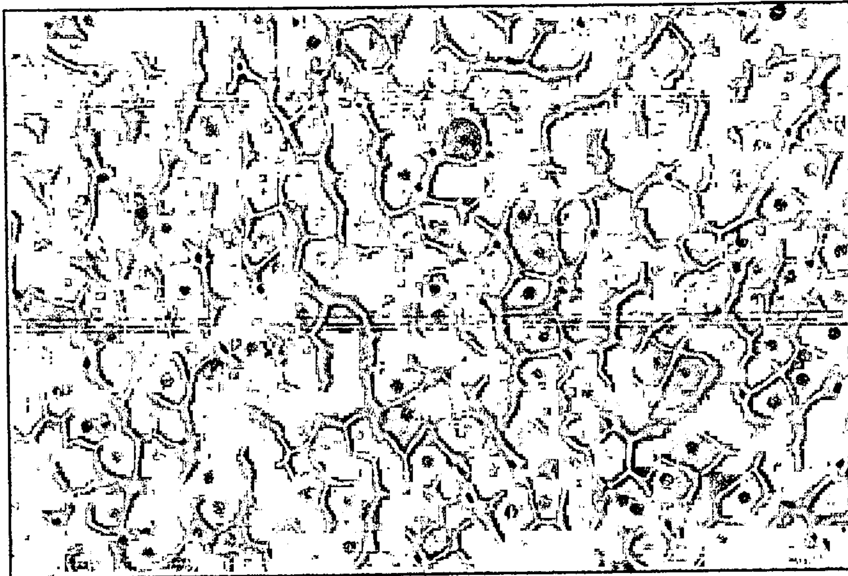


Figure 8.3 Histological characteristics of a rat liver in the control group, showing mild cellular hypertrophy. Liver enzymes for this specific animal were, in U/L; ALP 190, AST 155 and ALT 79.

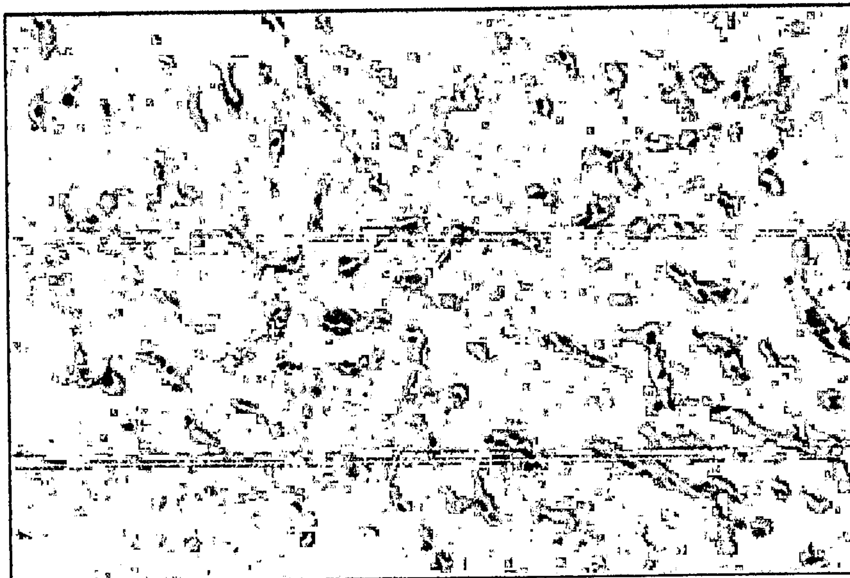


Figure 8.4 Histological characteristics of a rat liver in the 'nevirapine only' treated group, hepatocellular hypertrophy and apoptosis was detected. Liver enzymes of this specific animal were, in U/L; ALP 151, AST 175 and ALT 56.

8.3.4.4 Comment

Whereas the liver function tests of the 'nevirapine only' treated rats were not significantly different from the control group, indicating no hepatotoxicity, the histological findings of the 'nevirapine only' treated rats depicted hepatotoxicity. This therefore indicated that the diagnosis of liver injury could not be made solely on the changes in liver function enzymes. These findings also implied that nevirapine-induced hepatotoxicity was associated with enzyme induction.

Since the 'ketoconazole only' treated group did not exhibit hepatotoxicity, the hepatotoxicity observed when ketoconazole was administered together with nevirapine indicates that, either, nevirapine-induced hepatotoxicity might be worsened by ketoconazole, or ketoconazole became more toxic due to competition for metabolism. Ketoconazole and nevirapine are both metabolised by CYP3A. Also, from this, one can conclude that ketoconazole did not prevent the occurrence of hepatotoxicity. However, because ketoconazole is a known hepatotoxic agent (Rodriguez, 1995; Findor, 1998), coupled with the toxicity observed in the 'ketoconazole plus nevirapine' treated group, it was suggested that a lower dose (10 mg/kg) should be used in the subsequent experiment.

8.3.5 PART III: PRE-TREATMENT WITH DEXAMETHASONE

8.3.5.1 Experiment

Rats were divided into five groups (Group H, I, J, K and L) of five rats each, all groups were pre-treated with dexamethasone for three days 50 mg/kg/day (as previously determined), after which they were treated as follows:

Determining the hepatotoxic dose of Nevirapine.

On the fourth day Group H was treated with water (vehicle) orally (control group), while Group I was treated with nevirapine 1340 mg/kg (orally, in two divided doses, 6 hours apart). Both these groups were sacrificed on the fifth day (24 hrs later).

Determining safety of ketoconazole (inhibitor).

On the fourth day Group J and K were administered 10 mg/kg and 20 mg/kg ketoconazole (CYP3A inhibitor) i.p., respectively, followed 1 hour later by water (vehicle) instead of nevirapine. Both these groups were sacrificed on the fifth day (24 hrs later).

Testing the inhibitor in preventing the development of hepatotoxicity after nevirapine overdose.

On the fourth day, Group L was administered ketoconazole 10 mg/kg 1 hour before nevirapine 1340 mg/kg (orally, in two divided doses, 6 hrs apart) was given. Animals were sacrificed on the fifth day (24 hrs later).

8.3.5.2 Results

Nevirapine Hepatotoxicity.

There was more hepatotoxicity in the 'nevirapine only' treated group (Group I) than in the control group (Group H; Table 8.6). This is indicated by the raised AST and ALT levels, about 2 times that of the control (appendix E).

Table 8.6. Liver function tests of the control group (Group H) and the nevirapine only group (Group I).

	Control Group H	**Nevirapine only Group I**	**P value**
	Mean ± SD (n = 5)	Mean ± SD (n = 5)	
ALP	136.60 ± 15.31	169.20 ± 67.21	0.5476
AST	146.40 ± 10.43	239.25 ± 50.72	0.0159*
ALT	149.60 ± 39.66	386.00 ± 154.31	0.0317*

(Mann-Whitney U Test, 0.05 level of significance)

Safety of ketoconazole.

The liver enzymes of rats treated with the inhibitor, ketoconazole 10 mg/kg (Group J) were not significantly different from the control (Group H, Table 8.7). However, the AST level in rats treated with the higher dose (ketoconazole 20 mg/kg, Group K) was significantly different from the control (Table 8.8). It was therefore decided to use 10 mg/kg ketoconazole, for further experiments.

Table 8.7. Liver function tests of rats in the control group (Group H) and those treated with ketoconazole (10 mg/kg, Group J).

	Control Group H	Ketoconazole, 10 mg/kg Group J	P value
	Mean ± SD (n = 5)	Mean ± SD (n = 5)	
ALP	136.60 ± 15.31	142.80 ± 13.95	0.5476
AST	146.40 ± 10.43	150.20 ± 64.25	0.4206
ALT	149.60 ± 39.66	144.60 ± 39.18	0.8413

(Mann-Whitney U Test, 0.05 level of significance)

Table 8.8. Liver function tests of rats in the control group (Group H) and those treated with ketoconazole (20 mg/kg, Group K).

	Control Group H	Ketoconazole (20 mg/kg) Group K	P value
	Mean ± SD (n = 5)	Mean ± SD (n = 5)	
ALP	136.60 ± 15.31	121.40 ± 6.23	0.0556
AST	146.40 ± 10.43	257.20 ± 96.54	0.0159*
ALT	149.60 ± 39.66	229.60 ± 99.74	0.2222

(Mann-Whitney U Test, 0.05 level of significance)

Testing the inhibitor in preventing hepatotoxicity. The liver enzymes of the rats treated with 'ketoconazole and nevirapine' (Group L) were not significantly different from those treated with 'nevirapine only' (Group I, Table 8.9).

Table 8.9. Liver function tests of rats treated with 'nevirapine only' (Group I) and those treated with ketoconazole (10 mg/kg) and nevirapine (Group L).

	Nevirapine only Group I	Ketoconazole and nevirapine Group L	P value
	Mean ± SD (n = 5)	Mean ± SD (n = 5)	
ALP	169.20 ± 67.21	166.00 ± 66.43	0.8413
AST	239.25 ± 50.72	555.00 ± 239.62	0.0571
ALT	386.00 ± 154.31	406.60 ± 122.66	0.6905

(Mann-Whitney U Test, 0.05 level of significance)

Figure 8.5 illustrates the liver function tests of all the three groups (H, I and L). They confirmed that hepatotoxicity was achieved by pre-treating rats with dexamethasone prior to nevirapine dosing. From this figure, it is also clear that the use of a CYP3A inhibitor did not prevent nevirapine-induced hepatotoxicity, suggesting that CYP3A may not have a role in nevirapine-induced hepatotoxicity.

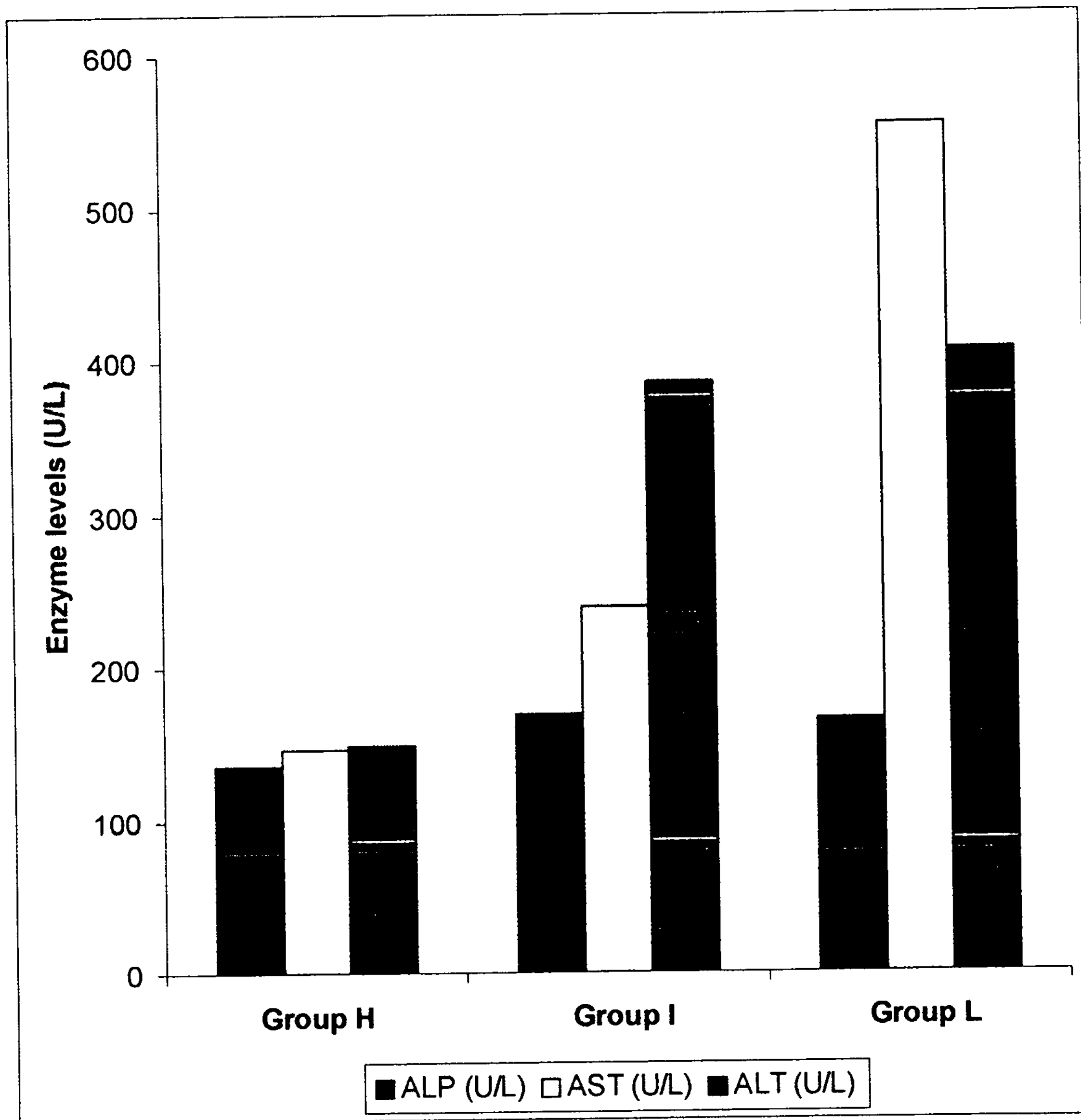


Figure 8.5 Liver function tests of rats in the control group (Group H), 'nevirapine only' treated group (Group I) and 'ketoconazole plus nevirapine' treated group (Group L).

8.3.5.3 Histological findings of rats pre-treated with dexamethasone.

By: Dr WS Botha, VetPath (Pretoria, South Africa).

The following groups were selected for histology: Control (Group H), 'nevirapine only' (Group I), 'ketoconazole only' (Group J) and 'ketoconazole plus nevirapine' (Group L). Below are the histopathology reports, as reported by the pathologist.

Control group (n=5): "All of the liver sections examined show mild to moderate hepatocellular degeneration with some vacuolation in the cytoplasm as well as granularity at what is usually recorded as cloudy swelling of the cytoplasm. In none of theses sections any vacuolar hepatopathy or severe vacuolization of the hepatocytes could be detected" (Fig. 8.6).

'Nevirapine only' treated group (n=5): "In the hepatic parenchymal cells three sections show mild hepatocellular degeneration while mild vacuolar hepatopathy could be detected in one of the livers sections. In all of the liver sections hypertrophic changes could be detected which vary from mild in one, to moderate in two and severe hypertrophy in two liver sections. Apoptosis was demonstrated in three of the livers" (Fig. 8.7).

'Ketoconazole only' treated group (n=5): "These liver biopsies resemble the morphological findings regarded in the control group, in that hepatocellular degeneration of mild to moderate degree could be detected and no vacoular hepatopathy, hepatocellular hypertrophy or apoptosis was present" (Fig. 8.8).

'Ketoconazole plus nevirapine' group (n=5): In three of the liver sections, the morphological changes resemble to a large extent those recorded in the 'nevirapine only' group, where the toxic drug was used while two of the sections appear similar to that from the control group" (Fig. 8.9).

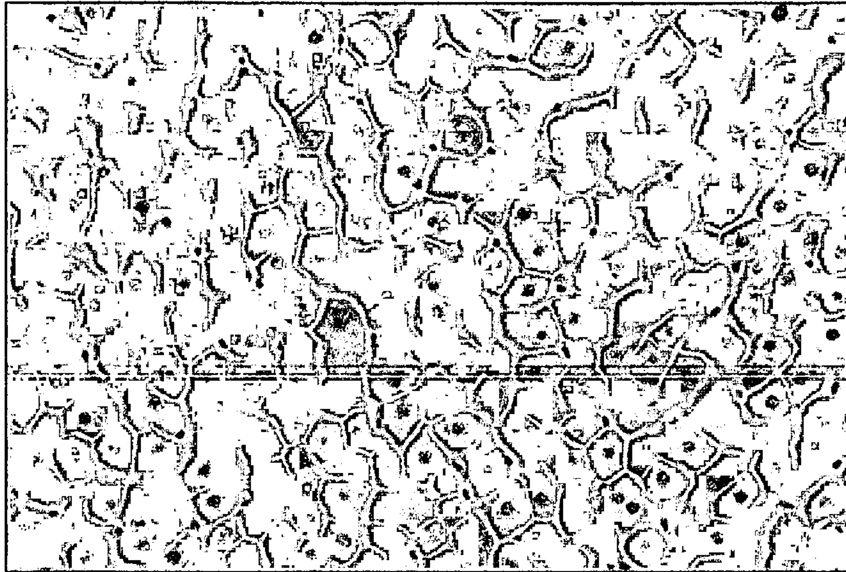


Figure 8.6 Histology of a rat liver in the control group, showing mild hepatocellular degeneration with some vacuolation in the cytoplasm. No vacuolar hepatopathy or severe vacuolization of the hepatocytes. Liver enzymes for this specific animal were, in U/L; ALP 121, AST 153 and ALT 211.

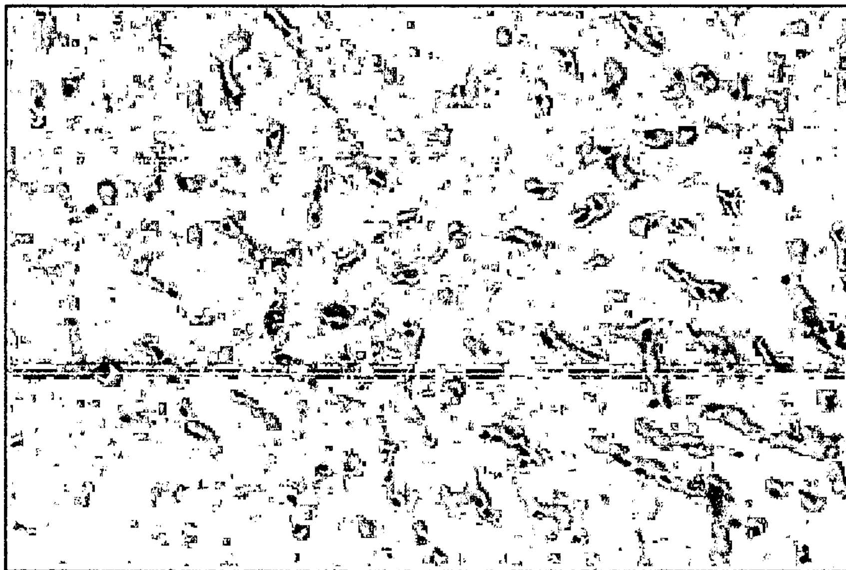


Figure 8.7 Histology of a rat liver in the 'nevirapine only' treated group, showing hepatocellular hypertrophy. Liver enzymes for this specific animal were, in U/L; ALP 144, AST 190 and ALT 112.

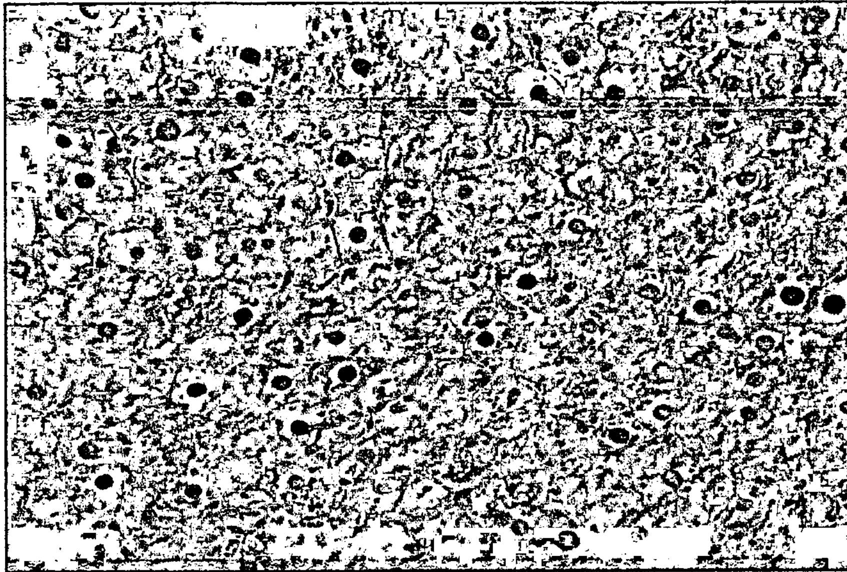


Figure 8.8 Histological characteristics of a liver treated with the inhibitor, showing similar findings as the vehicle group. No vacuolar hepatopathy, hepatocellular hypertrophy or apoptosis was detected. Liver enzymes for this specific animal were, in U/L; ALP128, AST 135 and ALT 158.

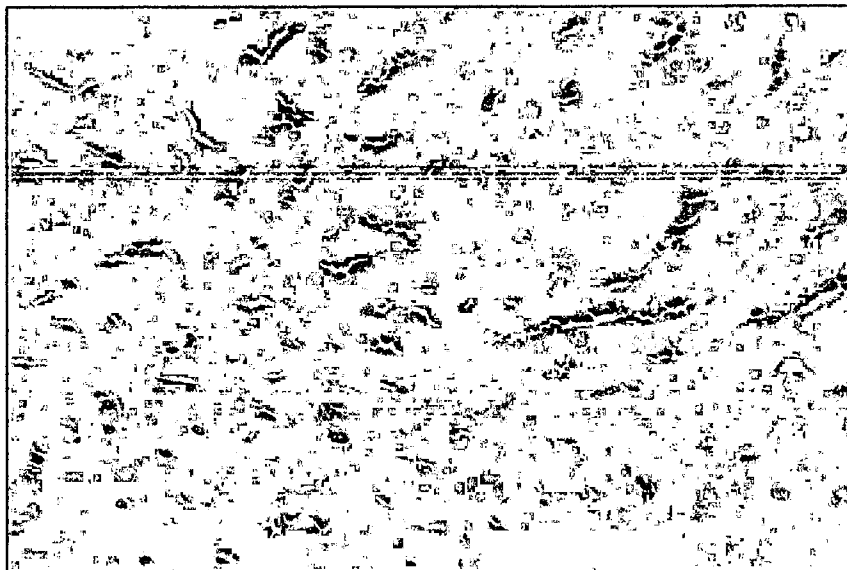


Figure 8.9 Histological characteristics of the liver treated with the inhibitor and nevirapine, hepatocellular hypertrophy and apoptosis was detected. Liver enzymes for this specific animal were, in U/L; ALP166, AST 620 and ALT 416.

8.3.5.4 Comment

According to the liver function tests as well as the morphological findings, nevirapine-induced hepatotoxicity was associated with enzyme induction by dexamethasone. Pre-treatment with dexamethasone probably resulted in an increased metabolic rate of nevirapine biotransformation, suggesting that hepatotoxicity might be due to metabolite accumulation in the liver.

However, the liver function tests and histology also indicated that the use of ketoconazole as a CYP3A inhibitor did not prevent the occurrence of hepatotoxicity. It was therefore suggested that CYP3A might not be responsible for this bioactivation.

Since CYP3A might not be involved in nevirapine-induced hepatotoxicity and because of nevirapine-induced hepatotoxicity being associated with enzyme induction by dexamethasone, it was thought that this enzyme inducer might have induced another isoform also involved in the metabolism of nevirapine. Therefore the possibility of another enzyme having a role in the metabolic activation of nevirapine exists, but further studies will have to be undertaken to ascertain this hypothesis.

CHAPTER 9

DISCUSSION

In this study, it was demonstrated that CYP3A may not be involved in nevirapine-induced hepatotoxicity and that nevirapine-induced hepatotoxicity is associated with enzyme induction. This is the first time such an observation has been reported as there was no report in the literature to which this scenario could be related other than the latent period (2-4 weeks) for toxicity to occur. Most reports on nevirapine-induced hepatotoxicity failed to associate it with enzyme induction but used the latter to explain nevirapine drug-interactions with other drugs such as rifampicin and the protease inhibitors (Mirochnick *et al.*, 2000; Reisler *et al.*, 2001; Boyle, 2002).

In light of the above observations, coupled with the mild hepatotoxicity after nevirapine overdose in the normal animals, the low or minimal CYP3A activity observed in these normal rats could reflect a similar pattern of low expression of the enzyme responsible for the hepatotoxicity. In this case, the changes in CYP3A activity were a good marker for the changes in the enzyme responsible for the hepatotoxicity. In other words, the inducers of CYP3A such as nevirapine and dexamethasone are also inducers of the culprit enzyme. This would then explain the increased hepatotoxicity observed after pre-treatment with nevirapine and dexamethasone. Dexamethasoe is a known inducer of CYP3A, CYP2B6 and CYP2C8 (Pascussi *et al.*, 2000; Schuetz *et al.*, 2000, Tredger and Stoll, 2002) while nevirapine is a known inducer of CYP3A and CYP2B6 (Morochnick *et al.*, 2000; Cheeseman *et al.*, 1993; Erickson *et al.,* 1999). Because CYP2B6 is induced by both drugs and it is known to activate drugs or chemicals into a toxic metabolite, e.g. cyclophosphamide (Code *et al.*, 1997; Schwartz and Waxman, 2001), one would conclude that it may be the possible culprit here. Most probably this could explain the increased toxicity when nevirapine was administered with ketoconazole (inhibitor of CYP3A and nevirapine 12-hydroxylation, Kishimoto *et al.*, 2000), in that ketoconazole could have inhibited the detoxifying pathway of nevirapine. Definitely, the role of CYP2B in nevirapine-induced hepatotoxicity needs to be established.

Nevirapine-induced hepatotoxicity, as indicated by the liver function tests, was more severe in rats pre-treated with dexamethasone than those pre-treated with nevirapine. This could be due to dexamethasone being a more potent enzyme inducer for the responsible enzyme than nevirapine. Of note, there was no significant difference in the activity of CYP3A between the two groups. On the other hand, this could be fictitious because liver enzymes were always higher in the dexamethasone than in the nevirapine pre-treated animals. This is important because, in the animals pre-treated with nevirapine followed by nevirapine overdose (nevirapine only group), the liver function tests (ALT 67.2 and AST 150.2) were lower than those of the corresponding group pre-treated with dexamethasone (ALT 386 and AST 239.3). Yet, the histopathology report was not different as it demonstrated hepatocellular hypertrophy in both groups. This may suggest that dexamethasone also induced the liver function enzymes and that the current liver function tests (AST and ALT) may not be a good marker for timely detection of nevirapine-induced hepatotoxicity. This concern has already been raised in clinical circles where a search for better markers of anti-retroviral induced hepatotoxicity has been instituted (personal communication from clinicians).

Whereas the pathology report indicated hepatocellular hypertrophy as the major hepatic lesion induced by nevirapine, direct observation of the representative sections in the photo's (Fig. 6.4 and 6.7) showed vivid pathology in the nuclei of the toxic animals compared to the control. There was granulation, disintegration and vacuolation in the nuclei of the toxic group, and therefore this could be an addition to the pathogonomic features of nevirapine-induced hepatotoxicity. Interestingly, these findings were similar to those described for monoacetylhydrazine (isoniazid metabolite) induced hepatotoxicity which also is by enzymatic metabolic activation (Walubo *et al.;* 1998).

CYP3A is known to be a male-predominant isoform in rats, as such, it was surprising to observe poor CYP3A activity in the male SD rats used in this study (Kedderis and Mugford, 1998). Of note, studies done earlier in this laboratory on rat CYP3A in this same specie (SD male rats), revealed adequate expression of CYP3A (Honours project). Then, the variation is likely to have occurred because of differences in the animal breed. This therefore indicates that variations in the expression of CYP3A

may exist among different breeds. Therefore, CYP3A expression, and probably other enzymes, should always be established before studies are conducted on animals.

The ketoconazole used to inhibit CYP3A is a non-selective enzyme inhibitor that is known to inhibit other enzymes such as CYP1A2 and CYP2C as well (Michalets, 1998). Then, the lack of protection against hepatotoxicity would imply that these isoforms might not be involved in nevirapine-induced hepatotoxicity. On the other hand, although it is well known that ketoconazole is potentially hepatotoxic, its mechanism of hepatotoxicity remains unclear, which made it difficult to determine whether it could have contributed to the toxicity observed when combined with nevirapine. As such a selective and well-characterised inhibitor should be used for all enzyme inhibitor experiments of this nature.

Lastly, it would have been valuable if the concentrations of the metabolites and nevirapine were measured. Unfortunately the company (Boehringer Ingelheim Pharmaceuticals, Inc., U.S.A) did not accept our request to supply the compounds and they were too expensive to secure from the open market.

CONCLUSIONS

1. A method for determining CYP3A activity *in vitro* was adapted using the erythromycin demethylation test. By this method, it was possible to determine the activity of CYP3A in normal rats and rats treated with nevirapine and dexamethasone. It was demonstrated that CYP3A activity was poor in normal rats and markedly increased in nevirapine and dexamethasone treated rats. It was concluded that nevirapine and dexamethasone were good enzyme inducers of CYP3A.

2. Nevirapine-induced hepatotoxicity was associated with enzyme induction by nevirapine and dexamethasone pre-treatment.

3. Liver function tests might not be a good marker for determining nevirapine-induced hepatotoxicity, as the liver enzymes did not correlate well with the histopathological findings of nevirapine-induced hepatotoxicity. Another marker should therefore be established for determining and assessing hepatotoxicity.

4. The pathogonomic features of nevirapine-induced hepatotoxicity included vacuolation, disintegration and granulation of the nuclei.

5. Ketoconazole (CYP3A inhibitor) did not prevent the occurrence of hepatotoxicity induced by nevirapine.

6. Therefore it was concluded that CYP3A might not have a role in the pathogenesis of nevirapine-induced hepatotoxicity.

CHAPTER 11

FUTURE STUDIES

1. There is a need to investigate the role of other CYP450 isoforms in nevirapine-induced hepatotoxicity.

2. The measurement of metabolites as well as the parent compound would be indicative of the actual metabolite that might be responsible for nevirapine-induced hepatotoxicity.

3. Determining an effective means or marker for assessing nevirapine-induced hepatotoxicity and to ascertain the standard pathogonomonic features related to nevirapine hepatotoxicity.

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APPENDICES

APPENDIX A

ERYTHROMYCIN DEMETHYLATION ASSAY

A-1 Standardisation of microsomal protein concentration

Tube #	µsomal protein mg/ml	usome	Buffer	Usomes mg/ml	Protein (mg)	µsomes 4 mg/ml	buffer (ul)	5 mM EDTA (ul)	100 mM MgCl (ul)	EM final Conc. mM	125 mM EM ul	NADP (ul)	CHO sol. (2.5 nmol/µl) (ul)	CHO nmol/ sample (0.5 ml)	CHO nmol/ml
Calibration															
1	20.025	199.75	800.25	4	0.4	100.00	290	20	20	5	20	50	0	0	0
2	20.025	199.75	800.25	4	0.4	100.00	280	20	20	5	20	50	10	25	50
3	20.025	199.75	800.25	4	0.4	100.00	270	20	20	5	20	50	20	50	100
4	20.025	199.75	800.25	4	0.4	100.00	260	20	20	5	20	50	30	75	150
5	20.025	199.75	800.25	4	0.4	100.00	250	20	20	5	20	50	40	100	200
6	20.025	199.75	800.25	4	0.4	100.00	240	20	20	5	20	50	50	125	250
Rxns															
7	12.069	497.14	502.86	6	0.1	16.67	290	20	20	5	20	50			
8	12.069	497.14	502.86	6	0.1	16.67	290	20	20	5	20	50			
9	12.069	497.14	502.86	6	0.2	33.33	290	20	20	5	20	50			
10	12.069	497.14	502.86	6	0.2	33.33	290	20	20	5	20	50			
11	12.069	497.14	502.86	6	0.3	50.00	290	20	20	5	20	50			
12	12.069	497.14	502.86	6	0.3	50.00	290	20	20	5	20	50			
13	12.069	497.14	502.86	6	0.4	66.67	290	20	20	5	20	50			
14	12.069	497.14	502.86	6	0.4	66.67	290	20	20	5	20	50			
15	12.069	497.14	502.86	6	0.5	83.33	290	20	20	5	20	50			
16	12.069	497.14	502.86	6	0.5	83.33	290	20	20	5	20	50			
17	12.069	497.14	502.86	6	0.6	100.00	290	20	20	5	20	50			
18	12.069	497.14	502.86	6	0.6	100.00	290	20	20	5	20	50			

A-2 Standardisation of time of incubation

Tube #	Time	µsomal protein mg/ml	usome	Buffer	usomes mg/ml	Protein (mg)	µsomes 4 mg/ml ul	buffer (ul)	5 mM EDTA (ul)	100 mM MgCl (ul)	EM final Conc. mM	125 mM EM ul	NADP (ul)	CHO sol. (2.5 nmol/µl) (ul)	CHO nmol/ sample (0.5 ml)	nmol/ml
Calibration																
1		20.025	199.75	800.25	4	0.4	100.00	290	20	20	5	20	50	0	0	0
2		20.025	199.75	800.25	4	0.4	100.00	280	20	20	5	20	50	10	25	50
3		20.025	199.75	800.25	4	0.4	100.00	270	20	20	5	20	50	20	50	100
4		20.025	199.75	800.25	4	0.4	100.00	260	20	20	5	20	50	30	75	150
5		20.025	199.75	800.25	4	0.4	100.00	250	20	20	5	20	50	40	100	200
6		20.025	199.75	800.25	4	0.4	100.00	240	20	20	5	20	50	50	125	250
Rxns																
7	2.5	12.069	331.43	668.57	4	0.4	100.00	290	20	20	5	20	50			
8	2.5	12.069	331.43	668.57	4	0.4	100.00	290	20	20	5	20	50			
9	5	12.069	331.43	668.57	4	0.4	100.00	290	20	20	5	20	50			
10	5	12.069	331.43	668.57	4	0.4	100.00	290	20	20	5	20	50			
11	7	12.069	331.43	668.57	4	0.4	100.00	290	20	20	5	20	50			
12	7	12.069	331.43	668.57	4	0.4	100.00	290	20	20	5	20	50			
13	10	12.069	331.43	668.57	4	0.4	100.00	290	20	20	5	20	50			
14	10	12.069	331.43	668.57	4	0.4	100.00	290	20	20	5	20	50			
15	15	12.069	331.43	668.57	4	0.4	100.00	290	20	20	5	20	50			
16	15	12.069	331.43	668.57	4	0.4	100.00	290	20	20	5	20	50			

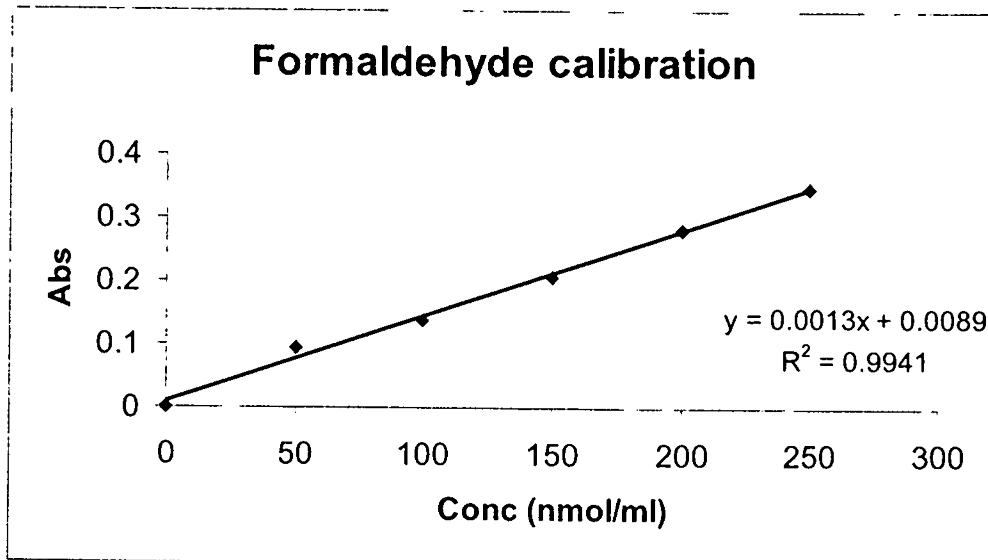
A-3 Standardisation of substrate concentration

Tube #	Substrate mM	µsomal protein mg/ml	usome	Buffer	usomes mg/ml	Protein (mg)	µsomes 4 mg/ml Ul	buffer (ul)	5 mM EDTA (ul)	100 mM MgCl (ul)	EM final Conc. mM	125 mM EM ul	NADP (ul)	CHO sol. (2.5 nmol/µl) (ul)	CHO nmol/ sample (0.5 ml)	nmol/ml
Calibration																
1		20.0	199.8	800.2	4	0.4	100	290	20	20	5	20	50	0	0	0
2		20.0	199.8	800.2	4	0.4	100	280	20	20	5	20	50	10	25	50
3		20.0	199.8	800.2	4	0.4	100	270	20	20	5	20	50	20	50	100
4		20.0	199.8	800.2	4	0.4	100	260	20	20	5	20	50	30	75	150
5		20.0	199.8	800.2	4	0.4	100	250	20	20	5	20	50	40	100	200
6		20.0	199.8	800.2	4	0.4	100	240	20	20	5	20	50	50	125	250
Rxns												EM = 25 mM				
7	1	12.1	331.4	668.6	4	0.4	100	290	20	20	5	20	50			
8	1	12.1	331.4	668.6	4	0.4	100	290	20	20	5	20	50			
9	2	12.1	331.4	668.6	4	0.4	100	270	20	20	5	40	50			
10	2	12.1	331.4	668.6	4	0.4	100	270	20	20	5	40	50			
11	5	12.1	331.4	668.6	4	0.4	100	290	20	20	5	20	50			
12	5	12.1	331.4	668.6	4	0.4	100	290	20	20	5	20	50			
13	10	12.1	331.4	668.6	4	0.4	100	270	20	20	5	40	50			
14	10	12.1	331.4	668.6	4	0.4	100	270	20	20	5	40	50			
15	20	12.1	331.4	668.6	4	0.4	100	230	20	20	5	80	50			
16	20	12.1	331.4	668.6	4	0.4	100	230	20	20	5	80	50			

A-4 Formaldehyde calibration for the standardisation of microsomal protein content

Calibration

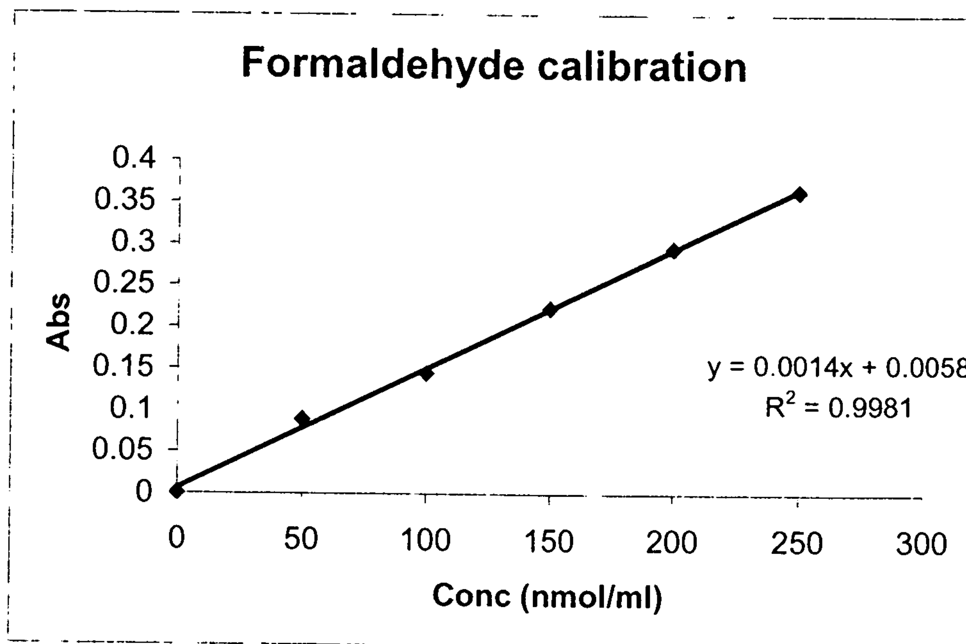
Conc nmol/ml	Abs	Net Abs
0	0.086	**0**
50	0.18	**0.094**
100	0.224	**0.138**
150	0.291	**0.205**
200	0.368	**0.282**
250	0.432	**0.346**



A-5 Formaldehyde calibration for the standardisation of time of incubation

Calibration

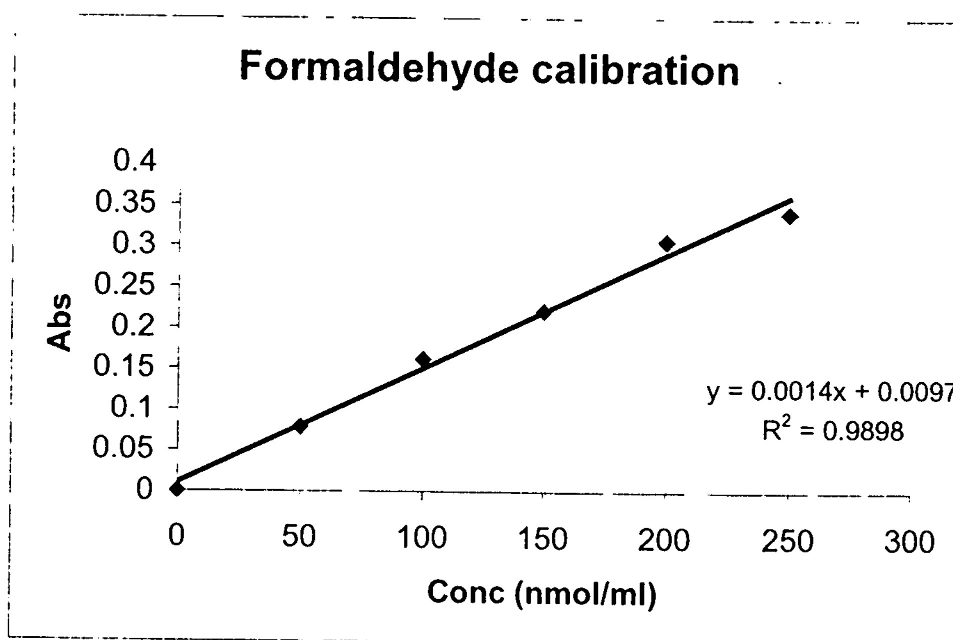
Conc nmol/ml	Abs	Net Abs
0	0.093	**0**
50	0.181	**0.088**
100	0.237	**0.144**
150	0.315	**0.222**
200	0.387	**0.294**
250	0.456	**0.363**



A-6 Formaldehyde calibration for the standardisation of substrate concentration

Calibration

Conc nmol/ml	Abs	Net Abs
0	0.108	**0**
50	0.186	**0.078**
100	0.269	**0.161**
150	0.328	**0.22**
200	0.414	**0.306**
250	0.448	**0.34**

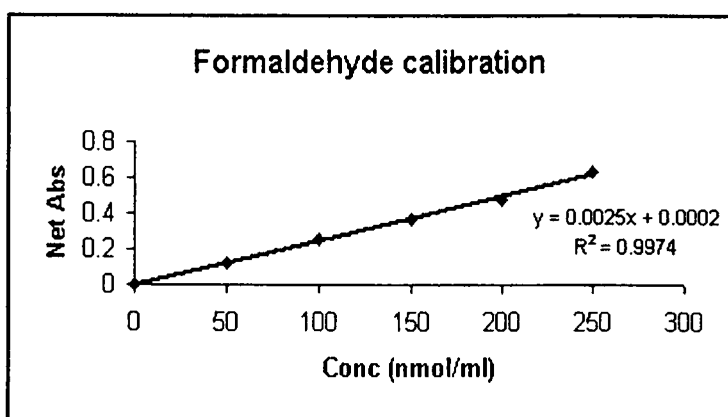


CYP3A ACTIVITY AFTER TREATMENT WITH DEXAMETHASONE AND NEVIRAPINE

B-1 Untreated rats

Calibration

Conc nmol/ml	Abs	Net Abs
0	0.12	**0**
50	0.245	**0.125**
100	0.373	**0.253**
150	0.49	**0.37**
200	0.593	**0.473**
250	0.752	**0.632**

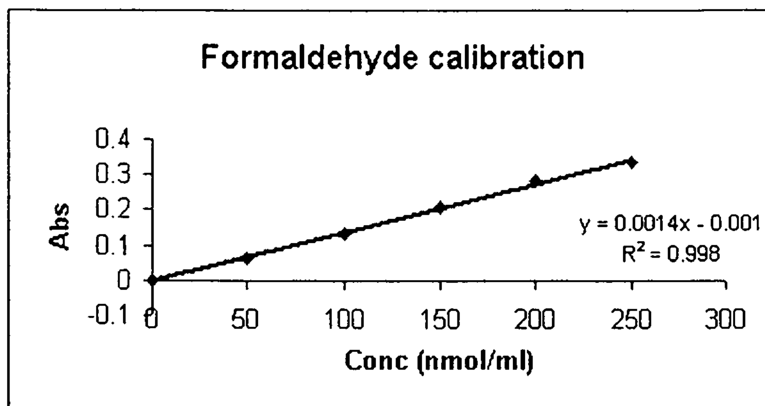


y = 0.0025x + 0.0002 Untreated rats (mM)	Abs	Net Abs	Conc nmol/ml	Average Conc	Nmol/sample	Velocity (nmol/min/mg)
1	0.122	0.002	0.72			
1	0.124	0.004	1.52	1.12	0.56	0.140
2	0.153	0.033	13.12			
2	0.131	0.011	4.32	8.72	4.36	1.090
3	0.142	0.022	8.72			
3	0.123	0.003	1.12	4.92	2.46	0.615
4	0.12	0	0.00			
4	0.161	0.041	16.32	8.16	4.08	1.020
5	0.123	0.003	1.12			
5	0.12	0	0.00	0.56	0.28	0.070

B-2 Dexamethasone 30 mg/kg/day for 3 and 5 days

Formaldehye calibration data

Conc nmol/ml	Abs	Net Abs
0	0.102	0
50	0.166	0.064
100	0.236	0.134
150	0.31	0.208
200	0.384	0.282
250	0.436	0.334

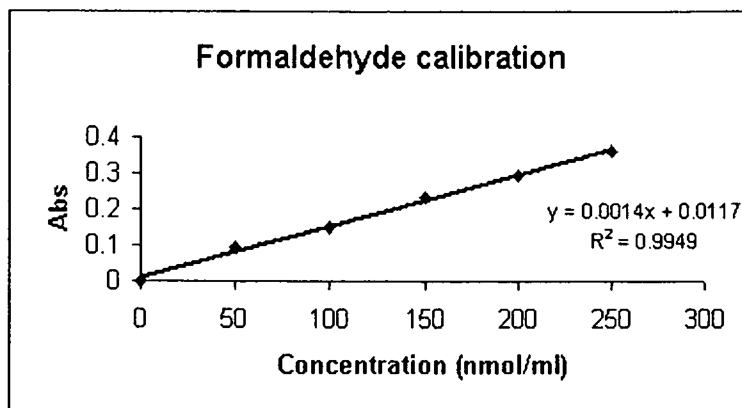


y = 0.0014x - 0.001	Abs	Net Abs	Conc nmol/ml	Average Conc	nmo	Velocity Nmol/min	Velocity Nmol/min/mg
Control	0.102	0	0.71				
Control	0.108	0.006	5.00	2.86	1.43	0.143	0.357
DEX (30mg/kg/day, 3 days)							
#1	0.141	0.039	28.57				
#1	0.134	0.032	23.57	26.07	13.04	1.304	3.259
#2	0.21	0.108	77.86				
#2	0.208	0.106	76.43	77.14	38.57	3.857	9.643
#3	0.191	0.089	64.29				
#3	0.183	0.081	58.57	61.43	30.71	3.071	7.679
#4	0.161	0.059	42.86				
#4	0.162	0.06	43.57	43.21	21.61	2.161	5.402
#5	0.189	0.087	62.86				
#5	0.209	0.107	77.14	70.00	35.00	3.500	8.750
DEX (30mg/kg/day, 5 days)							
#1	0.152	0.05	36.43				
#1	0.155	0.053	38.57	37.50	18.75	1.875	4.688
#2	0.196	0.094	67.86				
#2	0.206	0.104	75.00	71.43	35.71	3.571	8.929
#3	0.166	0.064	46.43				
#3	0.157	0.055	40.00	43.21	21.61	2.161	5.402
#4	0.162	0.06	43.57				
#4	0.162	0.06	43.57	43.57	21.79	2.179	5.446
#5	0.175	0.073	52.86				
#5	0.188	0.086	62.14	57.50	28.75	2.875	7.188

B-3 Dexamethasone 50 mg/kg/day for 3 and 5 days

ormaldehyde Calibration data

Conc nmol/ml	Abs	Net Abs
0	0.11	0
50	0.204	0.094
100	0.261	0.151
150	0.346	0.236
200	0.403	0.293
250	0.47	0.36

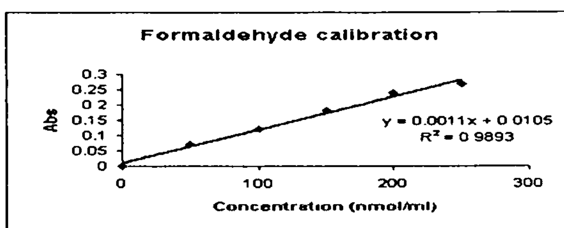


= 0.0014x + 0.0117	Abs	Net Abs	Conc nmol/ml	Ave. Conc	Nmol/ sample	Velocity Nmo/min	Velocity nmol/min/mg
ontrol	0.103	-0.007	0.00				
ontrol	0.109	-0.001	0.00	0.00	0.00	0.000	0
EX (50mg/kg/day, 3 days)							
1	0.232	0.122	78.79				
1	0.233	0.123	79.50	79.14	39.57	3.957	9.893
2	0.311	0.201	135.21				
2	0.297	0.187	125.21	130.21	65.11	6.511	16.277
3	0.204	0.094	58.79				
3	0.217	0.107	68.07	63.43	31.71	3.171	7.929
4	0.208	0.098	61.64				
4	0.196	0.086	53.07	57.36	28.68	2.868	7.170
5	0.248	0.138	90.21				
5	0.235	0.125	80.93	85.57	42.79	4.279	10.696
EX (50mg/kg/day, 5 days)							
1	0.233	0.123	79.50				
1	0.218	0.108	68.79	74.14	37.07	3.707	9.268
2	0.228	0.118	75.93				
2	0.226	0.116	74.50	75.21	37.61	3.761	9.402
3	0.216	0.106	67.36				
3	0.221	0.111	70.93	69.14	34.57	3.457	8.643
4	0.243	0.133	86.64				
4	0.254	0.144	94.50	90.57	45.29	4.529	11.321

B-4 Nevirapine 20 mg/kg/day and 40 mg/kg/day for 3 and 5 days

Calibration

Conc nmol/ml	Abs	Net Abs
0	0.122	0
50	0.192	0.07
100	0.242	0.12
150	0.305	0.183
200	0.36	0.238
250	0.389	0.267



y = 0.0011x + 0.0105	Abs	Net Abs	Conc nmol/ml	Average Conc	Nmol/sample	Velocity Nmol/min	Velocity nmol/min/mg
Control	0.125	0.003	-6.82				
Control	0.123	0.001	-8.64	0.00	0.00	0.000	0.000
NVP (20 mg/kg/day) 3 days							
#1	0.216	0.094	75.91				
#1	0.222	0.1	81.36	78.64	39.32	3.932	9.830
#2	0.168	0.046	32.27				
#2	0.166	0.044	30.45	31.36	15.68	1.568	3.920
#3	0.19	0.068	52.27				
#3	0.176	0.054	39.55	45.91	22.95	2.295	5.739
#4	0.219	0.097	78.64				
#4	0.223	0.101	82.27	80.45	40.23	4.023	10.057
#5	0.197	0.075	58.64				
#5	0.189	0.067	51.36	55.00	27.50	2.750	6.875
NVP (20 mg/kg/day) 5 days							
#1	0.192	0.07	54.09				
#1	0.203	0.081	64.09	59.09	29.55	2.955	7.386
#2	0.234	0.112	92.27				
#2	0.216	0.094	75.91	84.09	42.05	4.205	10.511
#3	0.199	0.077	60.45				
#3	0.21	0.088	70.45	65.45	32.73	3.273	8.182
#4	0.208	0.086	68.64				
#4	0.195	0.073	56.82	62.73	31.36	3.136	7.841
#5	0.178	0.056	41.36				
#5	0.195	0.073	56.82	49.09	24.55	2.455	6.136
NVP (40 mg/kg/day) 3 days							
#1	0.178	0.056	41.36				
#1	0.186	0.064	48.64	45.00	22.50	2.250	5.625
#2	0.154	0.032	19.55				
#2	0.158	0.036	23.18	21.36	10.68	1.068	2.670
#3	0.182	0.06	45.00				
#3	0.157	0.035	22.27	33.64	16.82	1.682	4.205
#4	0.155	0.033	20.45				
#4	0.158	0.036	23.18	21.82	10.91	1.091	2.727
#5	0.158	0.036	23.18				
#5	0.157	0.035	22.27	22.73	11.36	1.136	2.841
NVP (40 mg/kg/day) 5 days							
#1	0.165	0.043	29.55				
#1	0.17	0.048	34.09	31.82	15.91	1.591	3.977
#2	0.143	0.021	9.55				
#2	0.145	0.023	11.36	10.45	5.23	0.523	1.307
#3	0.177	0.055	40.45				
#3	0.179	0.057	42.27	41.36	20.68	2.068	5.170
#4	0.168	0.046	32.27				
#4	0.149	0.027	15.00	23.64	11.82	1.182	2.955
#5	0.156	0.034	21.36				
#5	0.161	0.039	25.91	23.64	11.82	1.182	2.955

APPENDIX C

LIVER FUNCTION TESTS OF NORMAL RATS

C-1 Control

Untreated

Rat#	1	2	3	4	5	Mean	SD	Median	Range
ALP	150	170	173	162	216	174.20	25.004	170	150 - 216
AST	81	92	97	129	110	101.80	18.431	97	81 - 129
ALT	76	170	93	104	97	108.00	36.159	97	76 - 170

C-2 Nevirapine only group

Rat #	1	2	3	4	5	Mean	SD	Median	Range
Weight (g)	318	344	316	325	321	324.80	11.26	321.00	316 - 344
ALP (U/L)	241	194	223	197	172	205.40	26.89	197.00	172 - 241
AST (U/L)	239	293	179	174	172	211.40	53.44	179.00	172 - 293
ALT (U/L)	133	89	91	71	87	94.20	23.09	89.00	71 - 133

C-2 Ketoconazole and Nevirapine group

Rat #	1	2	3	4	5	Mean	SD	Median	Range
Weight (g)	336	319	342	337	330	332.80	8.81	336.00	319 - 342
ALP (U/L)	172	187	219	179	182	187.80	18.27	182.00	172 - 219
AST (U/L)	154	149	153	284	239	195.80	62.08	154.00	154 - 284
ALT (U/L)	71	73	84	101	65	78.80	14.18	73.00	65 - 101

APPENDIX D

LIVER FUNCTION TESTS OF RATS PRE-TREATED WITH NEVIRAPINE

D-1 Control Group

Rat #	1	2	3	4	5	Mean	SD	Median	Range
Weight (g)	287	288	294	280	299	289.60	7.23	288.00	280 - 299
ALP (U/L)	274	266	301	190	212	248.60	46.01	266.00	190 - 301
AST (U/L)	126	107	112	155	99	119.80	21.99	112.00	99 - 155
ALT (U/L)	141	80	109	79	60	93.80	31.67	80.00	60 - 141

D-2 Ketoconazole only group

Rat #	1	2	3	4	5	Mean	SD	Median	Range
Weight (g)	321	330	322	323	337	326.60	6.80	323.00	321 - 337
ALP (U/L)	125	148	174	155	155	151.40	17.64	155.00	125 - 174
AST (U/L)	171	184	114	124	242	167.00	51.45	171.00	114 - 242
ALT (U/L)	108	104	74	100	128	102.80	19.37	104.00	74 - 128

D-3 Nevirapine only group

Rat #	1	2	3	4	5	Mean	SD	Median	Range
Weight (g)	328	350	316	319	328	328.20	13.31	328.00	316 - 350
ALP (U/L)	167	151	231	153	136	167.60	37.11	153.00	136 - 231
AST (U/L)	145	175	149	159	123	150.20	19.11	149.00	123 - 175
ALT (U/L)	74	56	75	60	71	67.20	8.64	71.00	56 - 75

D-4 Ketoconazole plus nevirapine group

Rat #	1	2	3	4	5	Mean	SD	Median	Range
Weight (g)	321	315	331	340	349	331.20	13.79	331.00	315 - 349
ALP (U/L)	204	225	198	256	174	211.40	30.85	204.00	174 - 256
AST (U/L)	138	182	228	598	148	258.80	192.86	182.00	138 - 598
ALT (U/L)	104	148	159	*471	109	130.00	27.58	128.50	104 - 159

*Values disregarded when the mean, SD, median and range were determined

APPENDIX E

LIVER FUNCTION TESTS OF RATS PRE-TREATED WITH DEXAMETHASONE

E-1 Control group

Rat #	1	2	3	4	5	Mean	SD	Median	Range
Weight (g)	311	322	341	348	311	326.60	17.13	322	311 - 348
ALP (U/L)	146	125	133	121	158	136.60	15.31	133	121 - 158
AST (U/L)	135	161	141	153	142	146.40	10.43	142	135 - 161
ALT (U/L)	134	161	137	211	105	149.60	39.66	137	105 - 211

E-2 Nevirapine only group

Rat#	1	2	3	4	5	Mean	SD	Median	Range
Weight (g)	358	331	349	362	333	346.60	14.15	349.00	331 - 362
ALP (U/L)	128	144	168	121	285	169.20	67.21	144.00	121 - 285
AST (U/L)	306	190	212	249	*1170	239.25	50.72	230.50	190 - 306
ALT (U/L)	374	*112	181	441	548	386.00	154.31	407.50	181 - 548

E–3 Ketoconazole 10 mg/kg

Rat #	1	2	3	4	5	Mean	SD	Median	Range
Weight (g)	340	348	331	350	338	341.40	7.73	340.00	331 - 350
ALP (U/L)	128	132	161	140	153	142.80	13.95	140.00	128 - 161
AST (U/L)	135	258	113	152	93	150.20	64.25	135.00	93 - 258
ALT (U/L)	158	143	103	203	116	144.60	39.18	143.00	103 - 203

E-4 Ketoconazole 20 mg/kg

Rat #	1	2	3	4	5	Mean	SD	Median	Range
Weight (g)	348	334	317	306	335	328.00	16.51	334.00	306 - 348
ALP (U/L)	131	117	115	123	121	121.40	6.23	121.00	115 - 131
AST (U/L)	216	156	414	268	232	257.20	96.54	232.00	156 - 414
ALT (U/L)	92	315	334	229	178	229.60	99.74	229.00	92 - 334

E-5 Ketoconazole plus nevirapine group

Rat #	1	2	3	4	5	Mean	SD	Median	Range
Weight (g)	360	341	346	337	326	342.00	12.47	341.00	326 - 360
ALP (U/L)	166	109	269	106	180	166.00	66.43	166.00	106 - 269
AST (U/L)	620	*63	827	250	523	456.60	302.45	523.00	63 - 827
ALT (U/L)	416	394	602	270	351	406.60	122.66	394.00	270 - 602

* Values disregarded when the mean, SD, median and range were determined

CONFERENCE ABSTRACT

Faculty of Health Sciences: Faculty Form 2003

THE ROLE OF CYP3A IN NEVIRAPINE INDUCED HEPATOTOXICITY

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Department of Pharmacology

Introduction and aim: Nevirapine is a potent non-nucleoside reverse transcriptase inhibitor with favourable pharmacokinetics that are characterised by rapid absorption and distribution with a long elimination half-life. Nevirapine is effective against HIV-1 when used in combination with other anti-retroviral agents and as a monotherapy for the prophylaxis of mother-to-child HIV-1 transmission. Unfortunately, the wide use of nevirapine is hampered by its adverse effects, mainly hypersensitivity skin reactions and hepatotoxicity. Since nevirapine induced hepatotoxicity commonly occurs between 2 – 12 weeks of treatment, and nevirapine is a known inducer of CYP3A isoenzyme, it was envisaged that the hepatotoxicity is due to activation of nevirapine to toxic metabolites by the induced enzymes. Therefore, the aim of this study was to determine the role of CYP3A in nevirapine induced hepatotoxicity.

Method: Three groups of five SD rats each were pre-treated with dexamethasone (50 mg/kg) intraperitoneally for three days to induce CYP3A. On the fourth day, Group A (control) was administered the vehicle, while group B was administered a toxic dose of nevirapine (1340 mg/kg), and Group C was administered ketoconazole (10 mg/kg) intraperitoneally, a CYP3A inhibitor, followed by the toxic dose of nevirapine one hour later. The rats were sacrificed 24 hours after treatment, blood was sent for liver function tests and the livers were saved for microsomal extractions and histology. Microsomal CYP3A activity was measured by the erythromycin demethylation test. Results of these animals were compared with results of animals that were not pre-treated with dexamethasone.

Results: Pre-treatment with dexamethasone lead to induction of CYP3A. CYP3A activity in the untreated group was 0.59 ± 0.48 nmol/min/mg versus 10.39 ± 3.59 nmol/min/mg after treatment. There was hepatotoxicity in group B, but hepatotoxicity was not prevented by ketoconazole in group C. Liver function tests were: ALP 169.2

± 67.21 U/L, AST 425.4 ± 418.56 U/L and ALT 386.0 ± 154.31 U/L for group B. and ALP 166 ± 66.43 U/L, AST 456 ± 302.45 U/L and ALT 406 ± 122.66 U/L for group C. Interestingly, there was no hepatotoxicity when the toxic dose of nevirapine was administered to animals that were not pre-treated with dexamethasone.

Conclusion: Nevirapine induced hepatotoxicity is associated with enzyme induction but CYP3A is not involved in its pathogenesis, and this suggests that a different enzyme may be responsible.

OPSOMMING

Nevirapien is 'n sterk nie-nukleosied trutranskriptase inhibeerder met gunstige farmakokinetika. Uitstaande eienskappe is vinnige absorpsie, distribusie en 'n lang half-leeftyd. Nevirapien is effektief teen HIV-1 as dit in kombinasie met ander antiretrovirale middels gebruik word en as dit as monoterapie vir die voorkoming van oordrag van moeder na baba. Ongunstige newe-effekte soos allergiese velreaksies en lewerskade beperk egter die gebruik. Aangesien nevirapien geïnduseerde lewerskade tussen 2 – 12 weke voorkom en nevirapien 'n bekende induseerder is, is die aanname dat die skade voorkom a.g.v. aktivering van nevirapien na toksiese metaboliete deur die geïnduseerde ensiem. Die doel van die studie was om vas te stel wat die rol van CYP3A is ten opsigte van nevirapine geïnduseerde lewerskade.

Vyftien manlike rotte is vooraf behandel met deksametasoon (50 mg/kg) of nevirapien (20 mg/kg) vir 3 dae. Op die vierde dag het die kontrole groep (n = 5) die draer ontvang, die 'nevirapien alleen' groep (n = 5) het 'n hoë dosis nevirapien ontvang (1340 mg/kg) en die 'ketokonasool en nevirapien' groep (n = 5) is behandel met 'n CYP3A inhibeerder (ketokonasool) een uur voor die hoë nevirapien dosering. Die diere is 24 uur later van kant gemaak en ontleed, plasma was gestuur vir lewerfunksie toetse, die lewer was verwyder vir mikrosoom ektraksies en histopatologiese studies. Mikrosomale CYP3A aktiwiteit is bepaal deur die eritromisien demetilering toets. Resultate van die groepe is vergelyk met die resultate van rotte wat nie met 'n induseerder vooraf behandel is nie.

Behandeling met deksametasoon of nevirapien het gelei tot verhoogde CYP3A aktiwiteit. CYP3A aktiwiteit in die onbehandelde, deksametasoon en die nevirapien groepe was (0.59 ± 0.48, 10.39 ± 3.59 en 7.28 ± 2.65 nmol/min/mg proteïen). In die deksametasoon vooraf behandelde groep was die histopatologie sowel as die verhoogde lewerensieme van die 'nevirapien-alleen' groep (AST 239.25 ± 50.7 U/L en ALT 386.00 ± 154.3 U/L) aanduidend van lewerskade in teenstelling met die kontrole groep (AST 146.40 ± 10.4 U/L en ALT 149.60 ± 39.7 U/L). Die ooreenstemmende groep wat vooraf met nevirapien behandel is, het egter geen betekenisvolle lewerensiem verhoging getoon nie (AST 150.20 ± 19.1 U/L en ALT

67.20 ±8.8 U/L). Die histopatologiese bevindinge het egter lewertoksisiteit getoon wat ooreengestem het met die van die deksametasoon groep. Lewertoksisiteit kon nie voorkom word in die groepe wat met ketokonasool behandel is voor die hoë dosis nevirapien nie. Interessante bevinding was dat geen lewerskade aangetoon kon word in die rotte wat oordoseer is met nevirapien sonder vooraf toediening van nevirapien of deksametasoon nie.

Samevattend, nevirapien geïnduseerde lewerskade is geassosieer met ensiem induksie deur deksametasoon of nevirapien. Die gebruik van ketokonasool het nie die lewerskade verhinder nie. CYP3A kan dus nie direk gekoppel word aan nevirapien verwante lewerskade nie en die moontlikheid dat 'n ander ensiem betrokke is verdien aandag. Aangesien die lewerfunksie toetse van die nevirapien vooraf behandelde groepe nie goed met die histopatologiese bevindinge gekorroleer het nie, word daar voorgestel dat lewerfunksie toetse alleen nie 'n goeie aanduiding is van lewerskade as gevolg van die toediening van nevirapien nie.

Sleutelwoorde: nevirapien, CYP3A, ketokonazool, deksametasoon, lewerskade, eritromisien demetilering toets en lewerfunksie toetse.

SUMMARY

Nevirapine is a non-nucleoside reverse transcriptase inhibitor that is used effectively to treat HIV-1 infection. As nevirapine-induced hepatotoxicity commonly occurs between 2 –12 weeks of treatment, and nevirapine is a known inducer of CYP3A, it was postulated that the hepatotoxicity was due to activation of nevirapine to toxic metabolites by the induced CYP3A. Therefore, the aim of this study was to determine the role of CYP3A in nevirapine-induced hepatotoxicity.

Fifteen male SD rats were pre-treated with either dexamethasone (50 mg/kg) or nevirapine (20 mg/kg) for 3 days. On the fourth day, the control group (n=5) was administered with the vehicle, while the 'nevirapine only' group (n = 5) was given an overdose of nevirapine (1340 mg/kg, orally) and the 'ketoconazole plus nevirapine' group (n = 5) was treated with a CYP3A inhibitor (ketoconazole) 1 hour before the overdose of nevirapine was given. The animals were sacrificed 24 hrs later and plasma was sent for liver function tests, the liver was excised for microsomal extraction and histopathology studies. Microsomal CYP3A activity was measured using the erythromycin demethylation test. Results of these animals were compared with results obtained in rats that were not pre-treated with an inducer before the toxic dose of nevirapine was administered.

Treatment with dexamethasone or nevirapine lead to increased CYP3A activity. CYP3A activity in the untreated, dexamethasone treated and nevirapine treated rats was 0.59 ± 0.48, 10.39 ± 3.59 and 7.28 ± 2.65 nmol/min/mg protein, respectively. In the dexamethasone pre-treated groups, the histopathological findings as well as the elevated liver enzymes of the 'nevirapine only' treated group (AST 239.25 ± 50.7 U/L and ALT ± 386.00 ± 154.3 U/L) were indicative of hepatotoxicity as opposed to the control group (AST 146.40 ± 10.4 U/L and ALT 149.60 ± 39.7 U/L). However, the corresponding group pre-treated with nevirapine did not show significant elevations in liver enzymes (AST 150.20 ± 19.1 U/L and ALT 67.20 ± 8.6 U/L) but the histopathological findings exhibited hepatotoxicity that was similar to the dexamethasone group. Nevirapine-induced hepatotoxicity was not prevented in the groups treated with ketoconazole before the overdose of nevirapine was given.

Interestingly, there was no hepatotoxicity when the overdose of nevirapine was administered to animals that were not pre-treated with nevirapine or dexamethasone.

In conclusion, nevirapine-induced hepatotoxicity was associated with enzyme induction by dexamethasone or nevirapine, and the use of ketoconazole did not prevent the hepatotoxicity. Therefore, CYP3A may not be involved in the pathogenesis of nevirapine-induced hepatotoxicity, suggesting that a different enzyme may be responsible. As the liver function tests did not correlate well with the histopathological findings in the nevirapine pre-treated groups, it was suggested that liver function tests alone might not be good markers for determining nevirapine-induced hepatotoxicity.

Key terms: nevirapine, CYP3A, ketoconazole, dexamethasone, hepatotoxicity, erythromycin demethylation and liver function tests.