

**Investigation into the hepatotoxic effects of
Highly Active Anti-Retroviral Therapy
(HAART) medications**

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Abstract

The development of the Highly Active Antiretroviral Therapy (HAART) for Human Immunodeficiency Virus (HIV) has greatly reduced the morbidity and mortality associated with HIV patients turning HIV infection into a chronic disease rather than a fatal one, through developing AIDS. The necessity for safe, efficacious, anti-retroviral agents is clear, however, long-term HAART has been linked to a wide range of complications including hepatotoxicity. Atripla and Eviplera, are one-pill-daily HAART regimens, containing the non-nucleoside reverse transcriptase inhibitors, efavirenz (1st generation) or rilpivirine (2nd generation) in combination with the nucleoside reverse transcriptase inhibitors emtricitabine, and tenofovir. Though these drug combinations are proposed to be safer than the older reverse transcriptase inhibitors the effects of these drugs on hepatocytes has yet to be fully elucidated. The overall objective of this thesis was to examine the effects of the components of Atripla and Eviplera on hepatocytes.

This project utilised the human hepatocyte cell line HepG2. Mitochondrial function was used as a measure of hepatocyte cell viability while morphological techniques were used to determine levels of apoptosis and necrosis. Oxidative stress was measured by dihydrodichlorofluorescein diacetate (DCF) fluorescence. Inflammation was assessed by hepatocyte production of the cytokine Interleukin (IL-8). The role of endoplasmic reticulum (ER) stress was determined by Western blotting for the protein marker, CHOP.

Neither tenofovir nor emtricitabine had any effect of hepatocyte cell viability, apoptosis or necrosis, and inflammation indicating that these components of Atripla and Eviplera are not hepatotoxic. However, both efavirenz and rilpivirine had hepatotoxic effects *in vitro*. Efavirenz dose-dependently caused a significant loss of HepG2 cell viability and increased levels of both apoptosis and necrosis that may be linked to the increased oxidative stress and ER stress observed with efavirenz exposure. Efavirenz caused a significant increase in inflammation as indicated by increased IL-8 release. The combination of efavirenz and the unsaturated fatty acid palmitic acid caused significant increase in cell death, IL-8 production and decreased cell viability compared to efavirenz or palmitic acid alone. Rilpivirine also dose-dependently reduced cell viability as well as increasing levels of both apoptosis and necrosis though not to the same extent as efavirenz. However, rilpivirine had a very interesting effect on IL-8 release with a biphasic effect where lower concentrations of rilpivirine decreased basal IL-8 release while higher concentrations stimulated IL-8 release.

In conclusion nucleoside reverse transcriptase inhibitors tenofovir and emtricitabine, have no hepatotoxic effects *in vitro*. Non-nucleoside reverse transcriptase inhibitors efavirenz and rilpivirine have direct toxic effects on hepatocytes decreasing cell viability and increasing inflammation. The 2nd generation drug rilpivirine, however, appears to have a better safety profile than the 1st generation drug efavirenz with a particularly novel effect on hepatic inflammation. efavirenz-mediated hepatotoxic effects can be enhanced in the presence of dietary saturated fatty acids such as palmitic acid suggesting that diet of HIV patients can have a significant impact on complications. Overall, transferring patients to Eviplera may reduce hepatic complications associated with HAART and, with the effect of rilpivirine on hepatic inflammation, may even have some hepatoprotective effects.

Abbreviations

ADP	adenosine diphosphate
AIDS	acquired immunodeficiency syndrome
ART	antiretroviral therapy
ATP	adenosine triphosphate
BHIVA	British Human immunodeficiency virus association
cART	combination Anti-retroviral therapy
CCR5	chemokine receptor type 5
cGMP	cyclic guanosine monophosphate
CHOP	CEBP homologous protein
cNOS	constitutive nitric oxide synthase
CXCR4	chemokine receptor type 4
DMEM	Dulbecco's Modified Eagle Medium
DMSO	dimethyl sulfoxide
DNA	deoxyribonuclease
EFV	Efavirenz
ER	endoplasmic reticulum
FCS	fetal calf serum
gp 41	glycoprotein 41
gp120	glycoprotein 120
HAART	highly active anitretorviral therapy
HDL	high density lipoprotein
HIV	human immunodeficiency virus
HPI	Hoescht/propidium iodide
Interleukin-8	IL-8
iNOS	inducible nitric oxide synthase
LDL	low density lipoprotein
MDA	malondialdehyde
mRNA	messenger ribonucleic acid
NAD+	nicotinamide adenine dinucleotide
NADPH	nicotinamide adenine dinucleotide phosphate
NAFLD	non alcoholic fatty liver disease

NASH	Nonalcoholic steatohepatitis
NF- κ b	Nuclear Transcription factor κ b
NNRTI	non nucleoside reverse transcriptase inhibitors
NO	nitric oxide
NRTI	nucleoside reverse transcriptase inhibitors
NtRTI	nucleoside reverse transcriptase inhibitor
O ₂ ⁻	superoxide
PBS	phosphate buffered saline
PI	protease inhibitors
PPAR- γ	Peroxisome Proliferator Activated Receptor gamma
RNA	ribonucleic acid
ROS	reactive oxygen species
RPV	rilpivirine
SDS	sodium dodecyl sulphate
SEM	standard error of the mean
SOD	superoxide dismutase
TBA	2-thiobarbituric acid
TNF- α	tumour necrosis factor-alpha
UPR	uncoupled protein response

Author's declaration

I declare that the research undertaken in this thesis, unless stated otherwise within the text, is the original work conducted by the author. This thesis has not been previously submitted before for a degree to this university or any other universities.

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Dated:

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Chapter 1

General Introduction

1.1 HIV/AIDS

1.1.1 History of Aids and HIV

Acquired Immune Deficiency Syndrome (AIDS) was described and identified in 1981 when a spike of patients in the United States presented with severe immunosuppression and common features to justify their explanations by clinicians to being a part of syndromic disease caused by a common pathogen. The causative agents were identified within a couple of years and abnormalities presented by the immune system resulted in the destruction of CD4⁺ T-lymphocytes, as a result the infected patient became immunologically compromised therefore the term Acquired Immune Deficiency Syndrome (AIDS) was officially recognised. Scientists and clinicians became aware that this disease has become a worldwide pathogen and also had the ability to infect almost every organ in the body and present severe illnesses especially in the advance stage of the disease (Coffin et al., 1986).

1.1.2 Epidemiology

There are two main types of the Human Immunodeficiency Virus (HIV) virus, HIV-1 and HIV-2. HIV-1 is more common in the western world and HIV-2 is more common in Africa, however the disease manifestations are similar (Castro-Nallar et al., 2012) An estimated 77,400 people were living with HIV in the UK at the end of 2007. It is now thought that there are over 90,000 people in the UK living with HIV and 33.4 million people living with HIV worldwide. The development of the Highly Active Antiretroviral Therapy (HAART) for HIV has greatly reduced the morbidity and mortality associated with patients developing AIDS and the rate of the development of HIV to AIDS has dramatically reduced (Abramowicz, 2006, Hammer et al., 2006, Egger et al., 2002). The necessity for safe, efficacious, anti-retroviral agents is clear. However, HAART has been linked to a wide range of side effects that are now becoming responsible for a renewed increase in morbidity and mortality rates of HIV positive patients with hepatotoxicity and renal damage being two of the major issues.

1.1.3 Transmission of HIV

The virus can be transmitted from vertical transmission from mother to child either through pregnancy, childbirth or breastfeeding, Horizontal transmission from contaminated blood, the sharing of needles and unprotected sexual intercourse.

HIV is transmitted by sexual contact, prenatally or by blood (most commonly by contaminated shared needles) (Tirelli et al., 1985).

1.1.4 The HIV life cycle and the Immune response

HIV is an enveloped RNA virus consisting of lipid and glycoproteins on the outer bilayer and an inner core consisting of two single RNA Strands, which are bound together by a gag-derived protein (p24). The outer bilayer is precisely structured and consists of elements that play a major role in the virus's infectivity and disease development. The viral envelope glycoprotein 120 (gp120) plays a major role as it interacts with the host cell receptors on healthy cells i.e. (macrophages, CD4⁺ lymphocytes, monocytes). Gp120 is closely linked with gp41, which is the envelope transmembrane viral protein necessary for viral-cell membrane fusion. Both are equally as important for infectivity (Fig1.1).

Gp120 attachment with the CD4⁺ receptor on the surface of the viable cells also involves chemokine co-receptors including CXCR4 (T-Cell-tropic) or CCR5 (macrophage-tropic), which enables cell binding and entry. Other minor chemokine co-receptors such as CCR1, CCR2, CCR3 and CCR4 may also play a part in the entry of the virus into CD4⁺ cells. Cellular events of the initial stage of attachment of the virus to the immune cells to the replication and budding of the new viruses from the cell leads to the invasion of substantial new viral particles, death of the infected cells which leads to the destruction of the immune system thus leading to the development of AIDS. (Gallo and Montagnier, 2003, He et al., 1997, Tien et al., 1999, Lee et al., 1998, Van-Rij et al., 1998)

Macrophages and dendritic cells that are present on the surface of mucus membranes bind to the HIV virus and carry them to the lymph nodes which have a high number of helper T cells, in this case other immune system cells are infected as well. Gp120 recognises and CD4⁺ protein and binds tightly onto the cells, p24 surrounds the

genetic materials of the virus (vital for infecting the cells) form a case around the cells that results in the production of new viruses. Without sufficient supply of helper T cells, the immune system cannot signal B cells to produce antibodies or cytotoxic T cells to kill the infected cells. The CD4⁺ and CCR5 membrane proteins are targets for HIV infection. Thus, the memory cells are rapidly infected and destroyed in the mucus membranes of our tissues. Also there are increased activated and unresponsive CD8⁺ T cells, increased β_2 -microglobulin and neopterin in serum, polyclonal B-cell activation with B cell refractory to T-cell-independent B-cell activators, and an increase in autoantibodies and immune complexes. This infection weakens the body's immune system, which cannot then fight off pathogens as well as before therefore lead to serious infections that don't often affect healthy people. These are called opportunistic infections (Beverley and Sattentau, 1987).

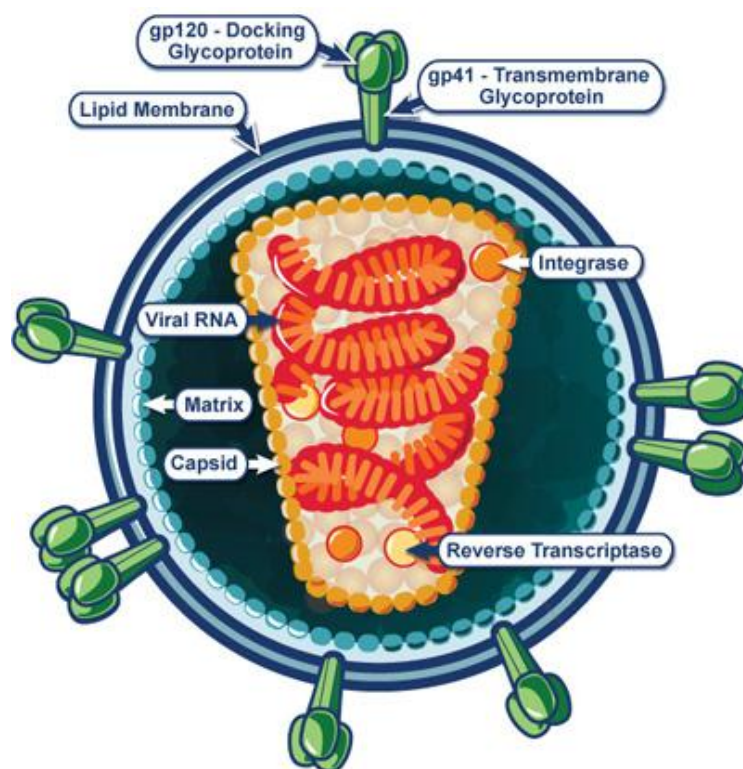


Figure 1.1 Anatomy of the Aids virus (<http://www.niaid.nih.gov/topics/hiv>)

The HIV lifecycle can be divided into six stages

Binding and Fusion: the beginning of the life cycle starts when the virus particle binds to a CD4⁺ receptor and with one or two co-receptors on the surface of a CD4⁺ T-lymphocyte. The virus fuses with the host cells releasing its RNA into the host cell.

Reverse Transcription: HIV enzyme (reverse transcriptase) converts the single-stranded HIV RNA to double-stranded HIV DNA.

Integration: The newly formed HIV DNA enters the host cell's nucleus, where an HIV enzyme called integrase "hides" the HIV DNA within the host cell's own DNA. The integrated HIV DNA is called provirus.

Transcription: When the host cell receives a signal to become active, the provirus uses a host enzyme called RNA polymerase to create copies of the HIV genomic material, as well as shorter strands of RNA called messenger RNA (mRNA). The mRNA is used as a blueprint to make long chains of HIV proteins.

Assembly: An HIV enzyme called protease cuts the long chains of HIV proteins into smaller individual proteins. As the smaller HIV proteins come together with copies of HIV's RNA genetic material, a new virus particle is assembled.

Budding: The newly assembled virus pushes out ("buds") from the host cell. During budding, the new virus steals part of the cell's outer envelope. This envelope, which acts as a covering, is studded with protein/sugar combinations called HIV glycoproteins. These HIV glycoproteins are necessary for the virus to bind CD4⁺ and co- receptors. The new copies of HIV can now move on to infect other cells (Figure 1.2)

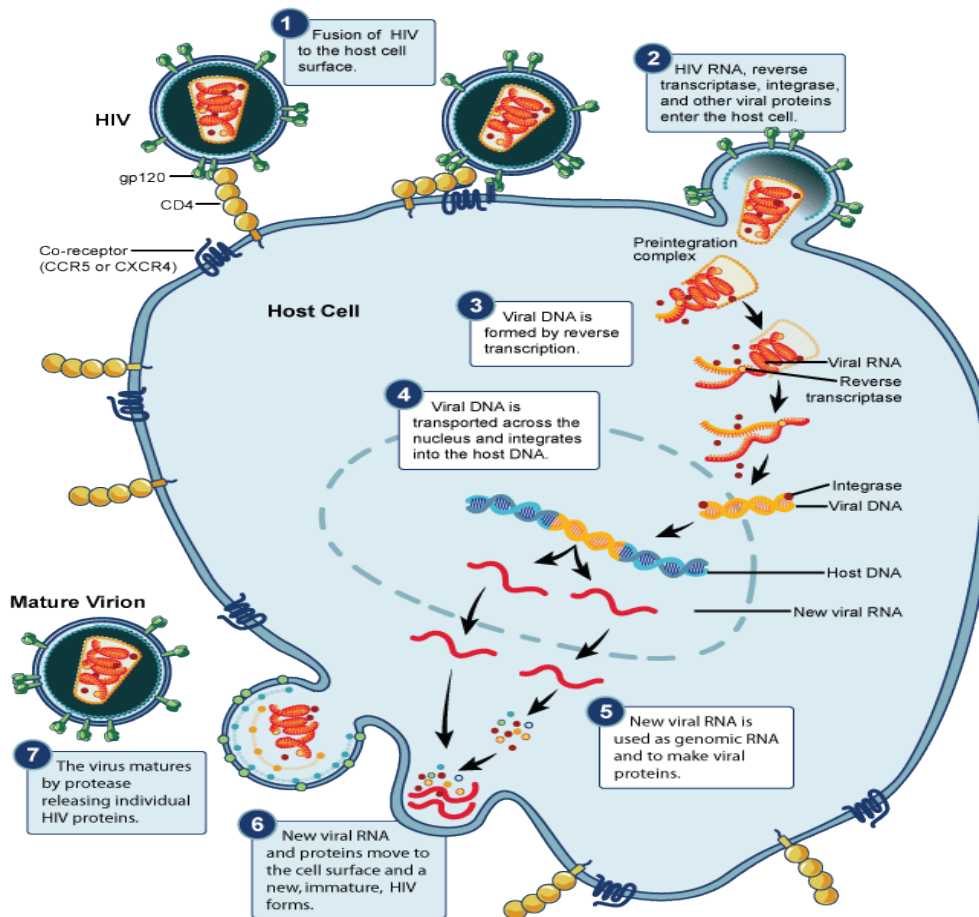


Figure 1.2 HIV replication cycle (<http://www.niaid.nih.gov/topics/hiv/aids>)

1.2 Treatment

1.2.1 Anti retroviral therapy

In recent years many new HIV antiretroviral drug classes have become available. There are five antiretroviral drug classes that are currently licensed for treatment of HIV-infected individuals; nucleoside/nucleotide reverse transcriptase (RT) inhibitors (NRTI), non-nucleoside reverse transcriptase inhibitors (NNRTI), protease inhibitors (PI), fusion and CCR5 inhibitors (FI and CCR5-I), and integrase inhibitors. In recent years many HIV antiretroviral drug classes have become available. These drug classes currently licensed for treatment of HIV infected people. These are included below with an example of a drug:

- Nucleoside reverse transcriptase inhibitors (NRTIs e.g. emtricitabine)

- Nucleotide reverse transcriptase inhibitors (NtRTIs e.g. tenofovir)
- Non-nucleoside reverse transcriptase inhibitors (NNRTIs e.g. efavirenz)
- Protease inhibitors (PIs e.g. ritonavir)
- Cell entry inhibitors (fusion inhibitors (FIs e.g. enfuvirtide))
- Co-receptor inhibitors (CRIs e.g. maraviroc)]
- Integrase inhibitors (INIs e.g. raltegravir) (De Clercq, 2009)

1.2.1.1 Nucleoside reverse transcriptase inhibitors (NRTIs)

The three classes of inhibitors including nucleoside RT inhibitors (NRTIs), nucleotide RT inhibitors (NtRTIs), and non-nucleoside RT inhibitors (NNRTIs) are all associated with the RT of HIV. The NRTIs and NtRTIs interact with the substrate-binding site of the enzyme, however the NNRTIs interact with an allosteric site at a short distance from the catalytic site.

The NRTIs and NtRTIs need to be phosphorelated to the triphosphate and disolphate form (an active form) in order to interact with the substrate-binding site.

NRTI are transformed into their active tri- or diphosphate (TP or DP) forms by cellular kinases. Structurally resembling the natural dNTPs, the active metabolites of NRTIs serve as alternative substrates for HIV-1 RT during viral DNA synthesis, which results in chain-termination of DNA elongation due to the lack of the 3'-hydroxy moiety (Feng et al., 2009). The Nucleoside Reverse Transcriptase Inhibitors (NRTIs) terminate chain elongation by acting as competitive inhibitors or alternate substrate with deoxycytidine-5'-triphosphate, the normal substrate in the reverse transcriptase reaction disabling further attachment of the normal substrate and thereby terminating chain elongation abruptly. NNRTIs are targeted at a binding site located in the palm domain of the p66 subunit of the reverse transcriptase, inducing allosteric changes in the enzyme as a result.

1.2.1.2 Nucleotide reverse transcriptase inhibitors (NtRTIs)

NtRTIs only need two phosphorelation steps to be converted to their active form. They contain a phosphonate group that cannot be cleaved by hydrolases making it more difficult to be cleaved off these compounds, once incorporated at the 3'-terminal end.

Non-nucleoside reverse transcriptase inhibitors (NNRTIs)

NNRTIs interact with an allosteric (non catalytic) site of the HIV RT. The binding site is at a close distance from the substrate (dNTP) binding site, disturbing the normal functioning of the RT. The amino acids that the NNRTIs interact with the binding pocket that may be prone to mutation.

NNRTI inhibit HIV-1 replication through multiple mechanisms, but mainly by inducing conformational changes within HIV-1 RT at the polymerase active site which significantly slow down viral DNA synthesis but have no effect on the binding affinity of natural dNTP and primer/template (Feng et al., 2009, Balzarini, 2004) They do not require intracellular phosphorylation to execute their antiviral action unlike the NRTIs, making them active against NRTI-resistant HIV strains. The active metabolites of Nucleotide Reverse Transcriptase Inhibitors (NtRTI) are phosphorylated before being incorporated at the DNA chain terminus through the phosphonate linkage. As a consequence, exonucleases that readily reverse the terminating effect of NRTIs by cleaving their monophosphates from the 3'-end of the DNA chain, fail to cleave NtRTIs.

1.2.1.3 Protease inhibitors (PIs)

They mimic the normal peptide linkage, which is cleaved by the HIV protease that cannot be cleaved therefore prevent the HIV protease from carrying out its normal functioning. Suppress further HIV infection by preventing proteolytic processing of precursor viral proteins into mature viral proteins.

1.2.1.4 Fusion inhibitors (FIs)

A polypeptide of 36 amino acids that is homologous to, and engages in a coil-coil interaction with, the heptad repeat (HR) regions of the viral glycoprotein gp41. Therefore blocking the fusion of the virus particle with the outer cell membrane. Inhibiting the processes necessary for the entry of the HIV into the host cell.

1.2.1.5 Co-receptor inhibitors (CRIs)

Prevent HIV attachment to the target cell via binding and antagonizing CCR5 receptor or CXCR4, which are used by macrophage tropic and T lymphocyte tropic HIV strains. Within the whole viral cell entry process, interaction of the viral glycoprotein gp120 with the co-receptor falls between the interaction of the viral glycoprotein gp120 with the CD4⁺ receptor and fusion of the viral glycoprotein gp41 with the outer cell membrane (Westby and van der, 2005).

1.2.1.6 Integrase inhibitors (INIs)

The HIV integrase has essentially two important catalytic functions (3'-processing and strand transfer) Figure 1.3 (De Clercq, 2009).

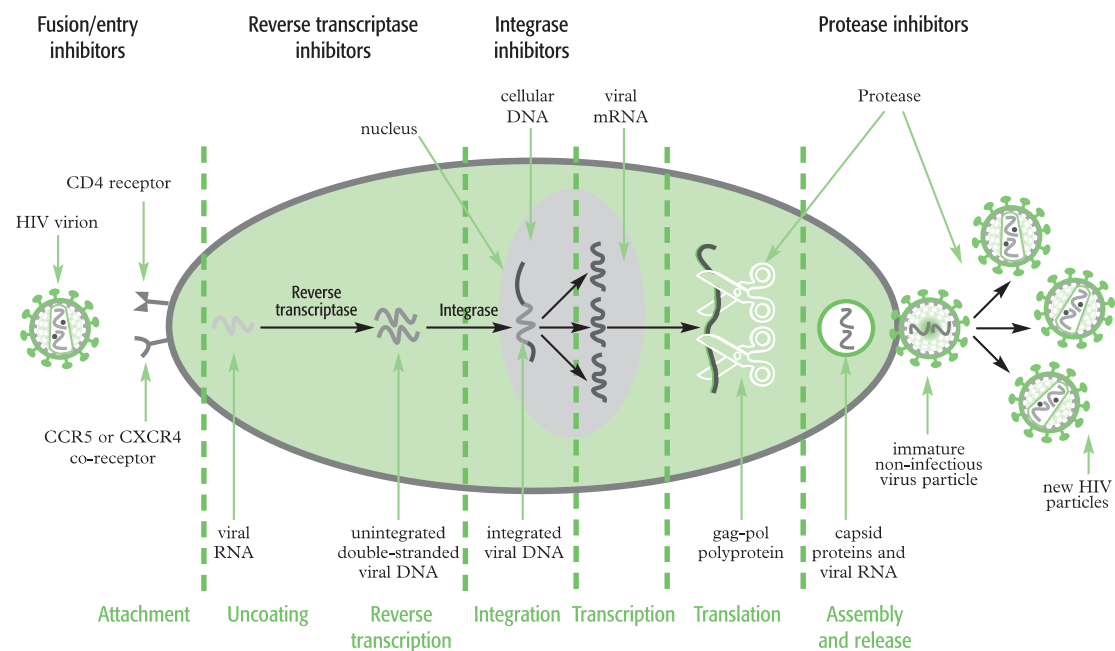


Figure 1.3 The mode of action of different classes of anti retroviral therapy (Adapted with kind permission of the National Institute of Allergy and Infectious Diseases).

1.3 Atripla and Eviplera

The combination of different drug classes have proven to be the most beneficial in terms of efficacy and long term safety in fighting the AIDS epidemic. Among all the drug classes the combination of nucleoside or nucleotide reverse transcriptase (RT) inhibitor (NRTI) and non-nucleoside RT inhibitor (NNRTI) have been the most extensively studied (Feng et al., 2009).

Atripla is currently recommended as first line treatment by BHIVA and is a combination of two NRTIs, emtricitabine and tenofovir and one NNRTI, efavirenz. Recently, the newly approved NNRTI has been compounded with emtricitabine, tenofovir and rilpivirine named Complera in the US and Eviplera in Europe. The action of each drug class is targeted at a specific stage of the HIV replication and life cycle, having two distinct mechanisms for reducing and eliminating the replicating virus (Bartlett and Lane, 2007).

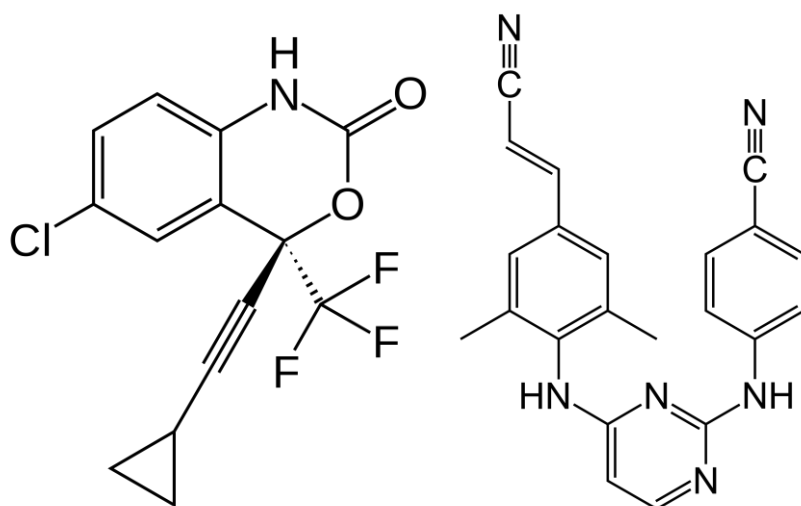


Figure 1.4 Structural formula of two NNRTIs efavirenz (left) and rilpivirine (right).

Emtricitabine is a synthetic nucleoside analog of cytidine, which has activity against HIV-1 RT as a nucleoside RT inhibitor (NRTI). It is phosphorylated by cellular enzymes to form emtricitabine 5'-triphosphate (Frampton and Croom, 2006). Activated emtricitabine inhibits viral RT by competing with deoxycytidine 5'-triphosphate (a natural substrate), incorporates into nascent viral DNA, resulting in chain termination and cessation of viral replication.

Tenofovir is an acyclic nucleoside phosphonate diester analog of adenosine monophosphate containing a phosphomimetic group. Tenofovir requires initial diester hydrolysis for conversion to tenofovir (NRTI) and subsequent diphosphorylations by cellular enzymes to form tenofovir diphosphate (Chapman et al., 2003, Dando and Wagstaff, 2004, Frampton and Croom, 2006). This is distinct from the other NRTIs in that those require triphosphorylation in order to become activated. Tenofovir diphosphate is the active component that then acts as a competitive inhibitor of the natural substrate (dATP deoxyadenosine 5'-triphosphate). Like emtricitabine, tenofovir is incorporated into nascent viral DNA and acts as a chain terminator, resulting in the cessation of viral replication. Each active component of Atripla inhibits the activity of HIV Reverse Transcriptase and results in the inhibition of viral replication (Lyseng-Williamson et al., 2005, Frampton and Perry, 2005, Young et al., 1995, Clay et al., 2008).

Efavirenz is widely used in the treatment of HIV-1 infection. Though highly efficient, there is growing concern about efavirenz-related side effects including metabolic disturbances and hepatic toxicity, the molecular basis of which remains elusive. Recent studies have demonstrated implications in stress responses involving mitochondrial dysfunction and oxidative stress in hepatic cells lines. ER-stress is associated with a growing number of liver diseases, including drug-induced hepatotoxicity. Adverse side effects frequently lead to a need to change the HAART therapy.

Rilpivirine is proposed to have an improved side effect profile in comparison to efavirenz, in addition it is more resilient to RNA polymerase mutations. However little evidence of *in vitro* toxicity exists. The two drugs are equally effective in terms of reducing viral load. Rilpivirine is a non-nucleoside reverse transcriptase inhibitor (NNRTI) recently developed as a drug of choice for initial antiretroviral treatment of HIV-1 infection. Disturbances in lipid metabolism and, ultimately, in adipose tissue

distribution and function are common concerns as secondary effects of antiretroviral treatment.

1.3.1 HAART Side effects

Although efavirenz, tenofovir and emtricitabine are reported to have minimal side effects their effectiveness in preventing HIV infected patients developing AIDs has reduced the mortality and increased the lifespan that adverse events are now being reported in patients. Efavirenz is known to cause lipid disturbances due to the alteration of lipid and fat composition, psychiatric symptoms and hepatic toxicity in a significant number of patients, lipid disturbances and hepatotoxicity due to inducing stress in hepatic cells by inhibiting mitochondrial function.

Following the introduction of highly active antiretroviral therapy (HAART), the life expectancy of HIV-infected patients has increased dramatically. HAART is a life-long therapy and a successful regimen is intended to be used for many years, the long-term side effects of these antiretroviral drugs are receiving increasing attention.

With prolonged exposure to antiretroviral drugs, several long-term side effects have been associated with the use of NRTI and their ability to inhibit polymerase- α the enzyme responsible for replication of mitochondrial (mt) DNA. mtDNA depletion in the liver may manifest as microvesicular steatosis, steatohepatitis or organ failure (Walker, 2002).

Although Atripla was developed as a HAART therapy for HIV infection with reduced side effects as compared to the older drugs, there have been some adverse effects reported by patients.

Efavirenz is associated with lipid disturbances and hepatotoxicity due to inducing stress in hepatic cells by inhibiting mitochondrial function.

Mitochondrial function interference leads to the accumulation of lipids in cytoplasm therefore activation of AMPK) Activated protein kinase.

The non-nucleoside reverse transcriptase inhibitors (NNRTIs) efavirenz are frequently used as components of current antiretroviral regimens. However, NNRTIs are known for their potential to cause hepatotoxicity, which can lead to morbidity and therapy switches. Different studies have reported a cumulative incidence of severe

hepatotoxicity. The debate regarding the pathogenesis of NNRTI- induced hepatotoxicity is ongoing. Hypersensitivity, mitochondrial toxicity, immune reconstitution and cumulative toxicity have been proposed as possible mechanisms, although a multifactorial cause is probably the most likely explanation (Van Welzen et al., 2012).

10% of patients treated with efavirenz have demonstrated raised liver enzyme serum levels associated with hepatic toxicity and may require treatment cessation with this particular agent. These adverse effects of the liver have been shown to be enhanced by saturated fatty acids which themselves cause fatty liver disease (Gao et al., 2010a, Ocama et al., 2010a).

Both tenofovir and emtricitabine have been associated with less side effects as compared to efavirenz. Tenofovir has been suggested to cause significant renal failure and osteoporosis. Emtricitabine appears to be the most free from toxicities however some lipid disturbances and depression has been reported by patients.

Liver toxicity is a frequent and significant occurrence in the setting of HIV infection that is treated with antiretroviral therapy, due to its intrinsic elevated frequency and its broad spectrum. Combined antiretroviral therapy may result in hepatotoxicity through a variety of direct and indirect mechanisms and associated disorders, such as mitochondrial toxicity, dyslipidemia and lipodystrophy syndrome, and steatohepatitis. Almost all antiretroviral drugs belonging to all available classes are responsible for an intrinsic liver toxicity, which is increased by the combined use of at least three different antivirals, in the so-called HAART (Manfredi et al., 2005).

With prolonged exposure to antiretroviral drugs, several long-term side effects have been associated with the use of NRTI and their ability to inhibit polymerase- α the enzyme responsible for replication of mitochondrial (mt) DNA. mtDNA depletion in the liver may manifest as microvesicular steatosis, steatohepatitis or organ failure (Walker, 2002).

Rilpivirine is a 2nd generation drug as compared to efavirenz and it was approved in 2011. To date very little basic investigations have been carried out with this drug so it is unknown whether this drug has deleterious effects on liver cells.

Liver toxicity is a frequent and significant occurrence in the setting of HIV infection that is treated with antiretroviral therapy, due to its intrinsic elevated frequency and its broad spectrum. Combined antiretroviral therapy may result in hepatotoxicity through a variety of direct and indirect mechanisms and associated disorders, such as

mitochondrial toxicity, dyslipidemia and lipodystrophy syndrome, and steatohepatitis (Perez-Molina, 2003, Milinkovic and Martínez, 2004, Sulkowski et al., 2002). Almost all antiretroviral drugs belonging to all available classes are responsible for an intrinsic liver toxicity, which is increased by the combined use of at least three different antivirals, in the so-called HAART (Manfredi et al., 2005).

1.4 Anatomy and physiology of the liver

The liver is the largest solid organ in the body. The liver receives venous blood straight from the gut and it's the first organ to receive nutrients and toxins from the intestine after absorption. One of the main roles of the liver is to detoxify the body from harmful substances and chemicals and metabolic by products. The liver has both exocrine and endocrine functions. Exocrine secretions are bile, which contains waste products and substances, which is required for intestine absorption. The endocrine function includes large amounts of secreted serum factors including albumin, prothrombin and protein components of lipoproteins. Other functions includes carbohydrate, urea and triglyceride metabolism.

The Glisson's capsule is a fibrous connective tissue, which surrounds the liver's four lobes. Approximately 80% of the liver cells consists of hepatocytes which carry out the main functions of the liver, these cells are organised into plates and are separated by sinusoidal capillaries (sinusoids). These sinusoids are lined by endothelial cells and macrophages known as Kupffer cells and are separated from hepatocytes by the space of Disse. The space of Disse is where Vitamin A is stored and fibroblast cells called Ito cells (lipocytes/stellate cells) can also be found here. The closeness of sinusoids and basal surface of hepatocytes help exchange substances between the blood and the liver cells. The blood entering the liver is from the hepatic portal vein and hepatic artery and the blood leaving the liver is from the hepatic vein. From this arrangement the liver can condition blood flowing from the intestine and pancreas to the rest of the body. The bile is collected by bile canaliculi, which then run between hepatocytes. The bile canaliculi converge to form bile ducts and are lined by biliary epithelial cells that channel bile towards the gall bladder.

The liver has many vital roles including carbohydrate metabolism, protein metabolism, protein synthesis, synthesis of clotting factors, blood cleansing, processing of drugs and toxins foreign to the body, fat metabolism and many more (Figure 1.5), (Duncan and Stephen, 2000).

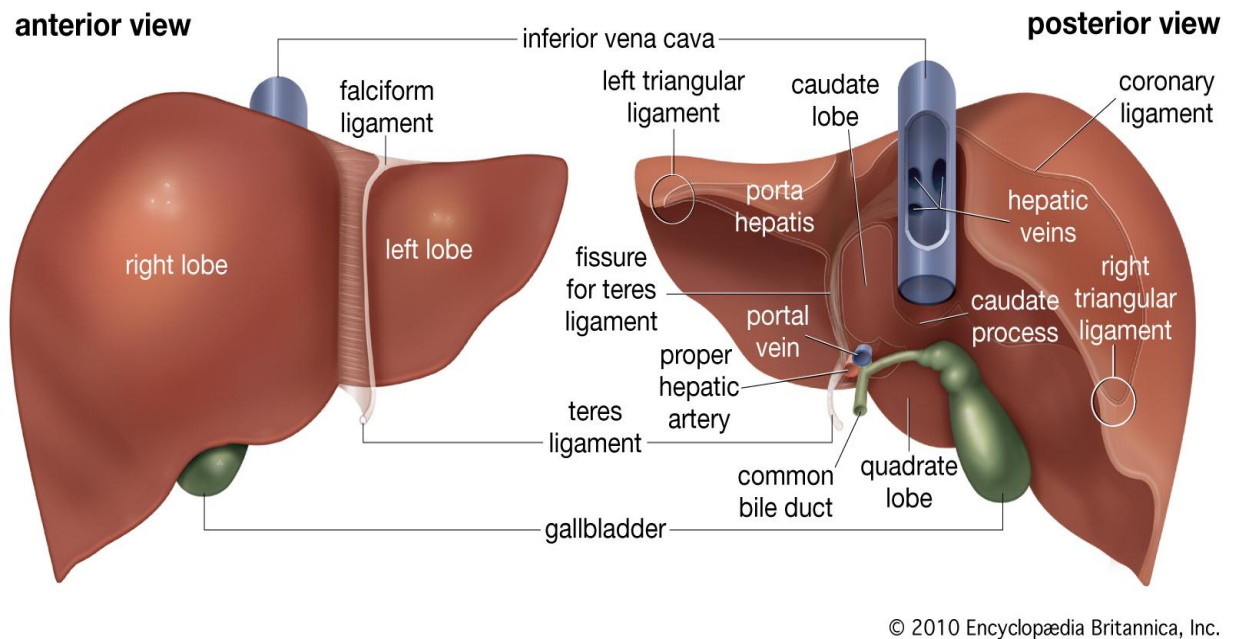


Figure 1.5 Anatomy of the Human Liver (<http://anatomisty.com/anatomy-systems/anatomy-of-human-liver/>)

1.4.1 Fat metabolism

One of the most important roles of the liver is the digestion of dietary fatty acid metabolism. The liver produces bile salts, which are essential for the emulsification of fatty acids within the intestines. Virtually all dietary fats are absorbed from the intestines into the lymphatic system. These fats are then broken into monoglycerides and fatty acids which are absorbed into the epithelial cells in the intestines which then enter the lymphatic system as small dispersed particles which are called chylomicrons.

Oxidation of fatty acids to supply energy Synthesis of cholesterol, phospholipids and lipoproteins Synthesis of fats from proteins and carbohydrates are metabolized in the liver. Fatty acid (palmitic acid) has 16 carbon atoms. The liver splits them into acetyl coA (two carbon atoms long) and this is done by beta-oxidation. The acetyl coA can enter the tricarboxylic acid cycle and be used directly as an energy source. Betaoxidation can take place in virtually in every cell in the body however the liver can produce more than it needs therefore being very efficient.

The excess acetyl coA is converted into the very soluble acetoacetic acid therefore passes easily into other cells in the body and is converted back to acetyl CoA and used as energy source. Some of the acetoacetic acid is converted into beta-hydroxybutyric acid and a small amount can be converted to acetone and can easy enter cells in the body and be used up for energy. Their presence in the blood is very low however in starvation mode the concentration of these three substances can enhance rapidly and their presence can exceed three times the normal amount and presence of acetoacetic can also be detected in the urine. Suggesting that fat is being used as an energy source.

The liver converts excess carbohydrates and protein into fatty acids and some of these will be stored in the liver and the rest will be released in the blood and then taken up into adipocytes and stored there as fat. Fatty acids are persistently cycling between the liver and adipose tissue (Hill, 2009).

1.5 Fatty acids

Fatty acids are important components of cell membranes are vital for intracellular signalling as precursors of ligands that bind to nuclear receptors 1-2. FAS are vital energy stores however raised levels of circulating free fatty acids (FFAs) contribute to complications such as obesity and metabolic syndromes by promoting excess fat deposition in non adipose tissues that are unsuitable for fat storage such as in the liver. Non alcoholic fatty liver disease (NAFLD) is thus hepatic manifestation of the metabolic syndrome. Fats include saturated and unsaturated fatty acids. In NAFLD patients the decreased unsaturated fatty acids in ratio to raised saturated fatty acids in serum and adipose tissue play a major role in the pathological syndromes.

The hepatocytes are not a physiological site for lipid storage and development of steatosis is associated with cellular dysfunction and apoptosis. Increased lipid storage in non-adipose tissues such as the heart and the liver are referred to as lipotoxicity and play a major role in tissue damage. Lipotoxicity is associated with reduced insulin sensitivity and inhibit signalling pathways and actions (Ricchi and et al., 2009).

Fatty acids are classified as saturated and unsaturated (monounsaturated and polyunsaturated) and their structure affects their biological effects. Fatty acids are carboxylic acids (-COOH) that contain hydrocarbon chains that range from four to 36 carbons long. Saturated fatty acids have no double bonds between their carbons of the macromolecule (e.g. palmitic acid). Monounsaturated fatty acids have single bonds or polyunsaturated fatty acids (PUFAs) have at least two double bonds somewhere along their hydrocarbon chain. Most of these fats are obtained from the diet and some can be synthesised in human tissues.

When dietary intake of fatty acids increases dramatically in the adipocyte capacity, the excess accumulate in non-adipose tissues therefore have pathological implications and can lead to apoptotic cell death (Reaven, 1988)

FFAs can induce the activation of a key regulator of inflammation nuclear factor kappa-light-chain-enhancer of activated B cells ($\text{NF}\kappa\text{B}$), as well as inflammatory cytokine tumour necrosis factor alpha, in hepatocytes while simultaneously causing c-Jun N-terminal kinases (JNK) dependant lipotoxicity.

1.5.1 Palmitic Acid

The lipotoxicity associated with palmitic acid has been shown to involve both ER stress and apoptotic cell death in the liver. The effects of palmitic acid are similar to tunicomycine, which is an inhibitor of protein glycosylation that promote ER stress.

The mechanisms of palmitic acid lipotoxicity involved in oxidative stress, altered mitochondrial permeability, changes in the morphology of ER and increased ER stress. It also has the ability of raising levels markers of ER stress such as p-elf2a and CHOP. CHOP is c/EBP homologous protein also known as growth arrest and DNA damage (GADD) 153 leading to cell death via apoptosis (Mabley et al., 2009).

By using such pathways, palmitic acid is able to induce apoptosis via activation of protein kinase C, $\text{NF}\kappa\text{B}$ or JNK. Prolonged and sever ER stress activates PERK-

mediated I κ B phosphorylation and leads to expression of activating transcription factor 4 (ATF4) and CHOP.

Interleukin 8 (IL-8) functions as a critical chemoattractant and activates neutrophils, basophils and T-cells. Elevated levels of IL-8 are associated with many diseases including obesity and various forms of liver injury. IL-8 may have significant roles in development of and progression of such diseases by causing inflammation and tissue injury. Hepatocytes have the capability of producing copious amounts of IL-8 therefore initiating and enhancing hepatic inflammation and injury. Patients with NAFLD and Nonalcoholic steatohepatitis (NASH) show to have significantly increased serum levels of IL-8. The exposure of hepatocytes to relevant pathophysiological concentrations of palmitic acid have shown to increase IL-8 levels (Joshi-Barve et al., 2003).

1.6 Fatty liver disease

Non-alcoholic fatty liver disease (NAFLD) is a disease that is predominantly caused by obesity and in the developed countries is the most cause of liver dysfunction. The prevalence of non-alcoholic fatty liver disease is around 20–30%, and with a rapid increase in the metabolic risk factors in the general population, non-alcoholic fatty liver disease has become the most common cause of liver disease worldwide (Martín-Carbonero et al., 2003).

NAFLD is considered the hepatic manifestation of insulin resistance (IR), and is therefore strongly associated with other clinical expressions of IR, such as metabolic syndrome and its features, namely obesity, type 2 diabetes, dyslipidemia and hypertension (Powell et al., 1990, Bugianesi et al., 2005). The pathogenesis implicates insulin resistance and increased oxidant stress with subsequent activation of collagen-making cells in the liver (Marchesini et al., 2001, Marchesini, 2003).

Increased hepatic free fatty acid (FFA) with obesity and insulin resistance derives the progression on NAFLD through direct hepatocyte lipotoxicity, oxidative stress with free radical production during fatty acid oxidation, ER stress, activated intracellular pathways such as NF κ B /IKK-B that promote and increase IR, cell injury, activates caspase dependent and independent necrotic and apoptotic cell death pathways and

ultimately leads to stellate cell activation and fibrogenesis and carcinogenesis which contribute to fat accumulation, NASH development and further progression (Anstee and Day, 2012).

1.6.1 Current knowledge of NAFLD pathogenesis

1.6.1.1 Genetic predisposition

Factors such as genetic predisposition together with environmental habits and diet play a major role in the pathogenesis of NAFLD. Some genes that effect fat accumulation, and also different mechanisms implicate the progression of the disease together with polymorphisms are capable of increasing the severity of NAFLD. For example genes implicate fat accumulation in the liver, adipokine/cytokine networks, oxidative stress and fibrogenesis.

1.6.1.2 Liver fat: mechanisms of hepatic accumulation and its role in the progression of the disease

In patients with NAFLD, liver fat derives from dietary FFA, liver FFA influx and lypogenesis are dependent on IR. Also a significant increase in the serum FFA in particular palmitate release from adipose tissue into plasma is seen in patients.

Dietary intake of NASH patients appear to contain high levels of saturated fat and cholesterol in relation to polyunsaturated fat.

It is worth noting that FFA derived from diet, and/or from adipose lypolysis and/or from de novo hepatic lipogenesis, not only are the ultimate substrate for the synthesis of liver triglycerides, but also have an important direct role in the pathogenesis of NAFLD. FFA interact with Toll like receptors (TLR), trigger cellular apoptosis, enhance oxidative stress, cytokine and ROS production and directly interfere with insulin signaling pathways (Malhi et al., 2006, Wang et al., 2006, Solinas et al., 2006).

1.6.2 NAFLD: risk factors and mechanisms of the disease

1.6.2.1 Bodyfat

Being overweight and obese are associated with NAFLD and the degree of obesity enhances the chances of developing NASH (Wanless and Lentz, 1990). However body mass index, central obesity and visceral fat are storage for FFA and therefore participate in the progression and severity of NAFLD in many ways, dependently and independently of IR, and also interfering with liver fat accumulation and progression from NAFLD to NASH (Chitturi et al., 2002, Thomas et al., 2005).

1.6.2.2 Oxidative stress and mitochondrial dysfunction

There is a strong correlation between severity of NASH and oxidative stress. As a result of excess FFA that undergo the oxidative process gives rise to oxidative stress in a way that is dependent on PPAR- γ . Mitochondrial oxidation is the leading pathway for the removal of fatty acids under normal physiological conditions, however in NAFLS this is increased and thus major source of reactive oxygen species (ROS) (Sanyal et al., 2001, Reddy and Mannaerts, 1994).

Patients with NASH have shown impairment in mitochondrial dysfunction. The dysfunction and alterations of the oxidative pathways in peroxisomes and microsomes increases ROS formations.

The relevance of the increase of these molecules arises from their ability to affect nucleotide and protein synthesis, to increase proinflammatory cytokine levels and to activate stellate cells (Esterbauer et al., 1991) leading to inflammation and liver fibrosis, thus progression from simple fatty liver to NASH.

1.6.2.3 Apoptosis

Hepatocyte apoptosis, via both extrinsic (receptor-mediated) and intrinsic (organelle-initiated) pathways, may play an important role in NAFLD pathogenesis and progression. Fas receptor overexpression in NASH patients shows a positive

correlation between hepatocyte apoptosis and hepatic fibrosis and inflammatory activity (Pettaa et al., 2009, Feldstein et al., 2003, Chiara et al., 2012).

1.7 Oxidative stress reactive oxygen species

The production of reactive oxygen species results in the damage to biomolecules for example lipids, proteins, amino acids and DNA. Free radicals including superoxides, nitric oxide and hydroxyl radicals and other reactive species e.g. hydrogen peroxide, proxynitrite and hypochlorous acid are produced in the body primarily as a result of aerobic metabolism.

Free radicals can be defined as molecules with an unpaired electron on the outer orbit. They are very unstable and thus very reactive.

Examples of oxygen free radicals are superoxide, hydroxyl, peroxy (RO_2), alkoxy (RO), and hydroperoxy (HO_2) radicals. Nitric oxide and nitrogen dioxide (NO_2) are two nitrogen free radicals. Oxygen and nitrogen free radicals can be converted to other non-radical reactive species, such as hydrogen peroxide, hypochlorous acid (HOCl), hypobromous acid (HOBr), and proxynitrite (ONOO). ROS, reactive nitrogen species (RNS), and reactive chlorine species are produced in humans under physiologic and pathological conditions (Evans and Halliwell, 2001) Thus, ROS and RNS include radical and non-radical species.

1.7.1 Biological Roles of Free Radicals

Oxygen radicals have critical actions in the body including signal transduction, gene transcription, and regulation of soluble guanylate cyclase activity in cells. Furthermore NO is an important signaling molecule. Physiological levels of NO that are produced by endothelial cells are vital for regulating the relaxation and proliferation of vascular smooth muscle cells, leukocyte adhesion, platelet aggregation, angiogenesis, thrombosis, vascular tone, and hemodynamics. Moreover, NO produced by neurons serves as a neurotransmitter, and NO generated by activated macrophages is very important mediator of the immune response. Conversely, as oxidants and inhibitors of enzymes which contain an iron-sulfur center, free radicals

and reactive species cause the oxidation of biomolecules for example protein, amino acids, lipid, and DNA, which leads to cell injury and cell death.

The cytotoxic effect of ROS produces many pathological effects on the human body on many levels including chronic diseases. Accordingly, there are two categories of free radicals, they serve as signaling and regulator molecules at physiologic levels but are highly deleterious and cytotoxic oxidants at pathologic levels (Fang et al., 2002).

Oxidative stress is the result of an imbalance in pro-oxidant/antioxidant homeostasis that leads to the generation of toxic reactive oxygen species (ROS). The importance of oxidative damage of tissue and cellular components as a primary or secondary causative factor in many different human diseases and aging processes. To cope with the oxidative stress, cells have developed a ubiquitous antioxidant defense system as a result of a number of external agents can trigger ROS production. An enzymatic and non-enzymatic antioxidant defense system including catalase (CAT), superoxide dismutase (SOD) glutathione peroxidase (GPx) and glutathione reductase together with a number of low molecular-weight antioxidants such as ascorbate, α -tocopherol and glutathione, cysteine, thioredoxin, vitamins counteracts and regulates overall ROS levels to maintain physiological homeostasis. (Fridovich, 1997).

1.7.2 Oxidative Stress and Liver

The liver is an important organ and is also an important place for free radicals. Liver enzymes, including diamine oxidase, aldehyde dehydro-genase, tryptophan dual oxidase, liver dehydrogenase and the cytochrome P450 enzyme system, induce oxidation and uncoupling (Irshad, 2002). Hepatocytes contain high levels of mitochondria thus produce excessive levels of unstable ROS. Additionally ER stress and peroxisomes have a large capacity to produce ROS than mitochondria, in the liver (Brown and Borutaite, 2012). Nitric oxide synthase is existent in liver parenchymal cells and Kupffer cells. Inflammation of the liver arises when nitrogen oxides exist in the form of free thiol with a reversible group through the role of glutathione-S-transferase (GSH-ST) (Diesen and Kuo, 2010). Nitrogen oxides can form reactive intermediates, such as nitro-cool acid, which produces necrotic cells in the liver, inhibition of mitochondrial function and consumption of cell pyridine nucleotides, which then leads to breakdown of DNA. Nitric oxide and peroxyion radicals

can combine to form hydrogen peroxide compound, causing inhibition of mitochondrial respiration, Na^+/K^+ pump function and the kinases phosphorylation, which can lead to cell damage. Overproduction of RNS can lead to nitrosylation reactions modifying the structure of proteins and consequently damage their regular functions (Klatt and Lamas, 2000).

Polyunsaturated fatty acids and hydrogen peroxide radicals act as intermediates of the formation of lipid peroxides on cell membrane (Niki, 2009). Moreover, human non-alcoholic liver disease show altered quantities of thio-barbiturates indicating high level of lipid peroxides in such patients (Stehbens, 2003). The activity of superoxide dismutases (SOD), glutathione peroxidase (GSH-PX) in chronic liver cirrhosis is significantly lower, which is negatively correlated with serum alanine aminotransferase (ALT) levels (Osman et al., 2007). Oxidative stress is closely related to the pathological damage of hepatic fibrosis (Rahman et al., 2012).

1.8 ER stress

The Endoplasmic Reticulum (ER) fulfils multiple cellular functions. A cell responds to, senses and avoids stress in order to maintain a normal homeostasis. There are many ways in which stress is manifested in a cell (endogenous and exogenous) including genetic mutation, infections, certain chemicals and oxidative stress. The protein folding process is very sensitive to such insults. Unfolded proteins can accumulate in the lumen of the ER and this leads to stimulated coordination of adaptive responses called the unfolded protein response (UPR). This process alleviates such stresses by up-regulating protein folding and degradation pathways in the ER therefore inhibiting protein synthesis.

ER is divided into rough and smooth ER. Smooth ER is in charge of carbohydrate and lipid synthesis, producing enzymes to detoxify compounds in the liver (Rutkowski and Kaufman, 2004).

Rough ER on the other hand is involved in the production of proteins. ER stress or UPR is mediated through three trans-membrane receptors including-

- The involvement of attenuation of protein synthesis through pancreatic ER Kinase (PERK) and ER Kinase.

- The activation of transcription factor 6 (ATF6), which is a transcription factor that induces ER chaperone production, that are capable of preventing protein accumulation.
- Inositol requiring enzyme 1 (IRE1) which induces many ER chaperones

These receptors are capable of halting the accumulation of proteins and allowing the elimination of unfolded proteins. However when adaptation fails ER initiated pathways signal and activates the $\text{NF}\kappa\text{B}$, which is a transcription factor that induces expression of genes encoding mediators of host defence. Excessive and prolonged stress triggers cell suicide (apoptosis).

ER chaperones GRP78, Binding immunoglobulin protein (Bip) prevent aggregation. However as the accumulation of proteins persists, GRP78 is then released which then launches UPR. PERK, Ire1 and ATF6 are critical trans-membrane ER signalling proteins. An alarm response is then produced and JNK is activated which are kinases linked to $\text{NF}\kappa\text{B}$. Finally as a last resort the cells induces apoptosis (Xu et al., 2005).

1.9 Aims

This study was carried out to evaluate the hepatotoxic effects of Atripla and Eviplera components, tenofovir, emtricitabine, efavirenz and rilpivirine on hepatic cell function and also how efavirenz can enhance the damaging effects of palmitic acid on HepG2 cell line. Cell viability, oxidative stress, cell death, IL-8 production and ER stress were evaluated with various concentrations in order to evaluate the hepatotoxic effects.

Chapter 2

Materials and methods

2.1 Materials

Abcam (Cambridge, UK)

B-Actin primary antibody mouse monoclonal IgG1 100µg/ml

GADD 153 primary antibody mouse monoclonal IgG1, 200µg/ml

Mouse anti-actin and goat anti-mouse secondary antibody

Axxora (Nottingham, UK)

BAY 11-7082

Quinazoline (QNZ)

BD Biosciences (Oxford, UK)

IL-8 assay kit

BioRad (Hertfordshire, UK)

β-mercaptoethanol

Laemmli Sample Buffer

European Collection of Cell Cultures (ECACC)

HepG2 Cell line

Fischer Scientific (Horsham, UK)

General laboratory grade chemicals

Tissue culture plastics

GE Healthcare (Hertfordshire, UK)

ECL plus Western Blotting Detection System

Fetal calf serum (FCS)

Molecular Weight Marker - Precision Plus Protein Standards

Non-essential amino acids

Penicillin/streptomycin

Phosphate buffered saline (PBS)

RPMI 1640 culture media

Protran Nitrocellulose transfer/blotting membrane

Protease inhibitor cocktail

Trypsin

Oxoid (Basingstoke, UK)

PBS tablets

Sequoia Research (Pangbourne, UK)

Efavirenz

Emtricitabine

Rilpivirine

Tenofovir

Sigma Aldrich (Poole, Dorset, UK)

2-Thiobarbituric acid

APS (ammonium persulfate)

dichlorofluorescein (DCF)

Dimethyl sulfoxide (DMSO)

Hoechst 33342 dye

Malonaldehyde bis Dimethyl acetal

Nitro blue tetrazolium (NBT)

Palmitic Acid

Propidium iodide

SDS (sodium dodecyl sulphate)

Tetramethylethylenediamine (TEMED)

Tetrazolium dye (MTT)

Trizma base

Tween 20

2.2 Buffers

Radioimmunoprecipitation assay buffer (RIPA)

150 mmol/L NaCl,
50 mmol/L Tris-HCl (pH 7.4)
1 mmol/L EDTA
1 mmol/L phenylmethanesulphonylfluoride (PMSF)
1% (v/v) Triton-X 100
1% sodium deoxycholate
0.1% (w/v) SDS

PBS- Phosphate Buffered Saline

2L distilled water
20 x PBS tablets obtained from (OXoid)

10x TBS (PH 7.6)

24.2g trizma base (tris) and 80 sodium chloride dissolved in 1L of water

Transfer buffer

100mls 10x running buffer, 200 mls methanol and 700 mls water.

10x running buffer

10g SDS lauryl sulphate, 30g trizma, 145g glycine, 1L water

Separating buffer (PH 8.8)

91g trizma 500mls water

Stacking buffer (PH 6.8)

30.5g 500mls water

Tween TBS

100 mls of 10x TBS
900mls water
500ul tween 20.

Running gel

3.3mls Acrylimide (30%)

4.3mls water

2.5mls separating buffer (tris 1.5M)

100ul 10% SDS

100ul 10% APS (100mg ammonium persulphate in 1ml water)

14ul TEMED

Stacking gel

830ul Acrylimide (30%)

2.8mls water

1.3mls stacking buffer (tris 0.5M)

50ul 10% SDS

50ul 10% APS (100mg ammonium persulphate in 1ml water)

7ul TEMED

2.4 Methods

2.4.1 Cell Culture

For the purpose of this investigation, cancerous human hepatocyte cell line, HepG2 were used. The HepG2 cells were passaged twice a week at subconfluent level and seeded at a density of 3.5×10^4 cells per well in 96-well plates for the MTT assay and 14×10^4 cells per well in a 48-well plate for the measurement of IL-8 assay depending upon each experimental protocol, and in 80 cm² flasks (1.2×10^5 cells/flask) seed density of 2.51×10^4 cells for maintenance. The culture used was RPMI-1640 media containing 10% fetal calf serum (FCS), supplemented with 1% penicillin and streptomycin, for treatment the FCS percentage was reduced to 3%. The cells were incubated at 37°C in a humidified atmosphere of 95% O₂ and 5% CO₂.

2.4.2 Experimental treatment

HepG2 cells were cultured and plated at the appropriate densities in appropriate plates with regards to each experiment (48, 96 well plates) prior to experiments in experimental media RPMI with 3% FCS to determine cell viability, inflammation, oxidative stress and ER stress. The cells were then treated with emtricitabine, tenofovir, efavirenz, rilpivirine and palmitic acid.

2.4.2.1 Palmitic Acid

Palmitic acid was prepared as a conjugate to fatty acid-free albumin, as palmitic acid itself acts as detergent and is toxic extracellularly. Conjugated to albumin, palmitic acid can cross cell membranes and exert its effects on the intracellular environment.

27.8mg of palmitic acid (20mmol/L) palmitic acid was prepared with 5mls of water, 200ul of sodium hydroxide (0.1 NAOH) and 200ul of ethanol. The mixture was then boiled at 80 °C for 5 minutes until palmitic acid was dissolved. 1 mL of the above mixture was then mixed with 1 mL 6 mmol/L albumin yielded a 10 mmol/L, which was then diluted for treatment of cells.

2.4.3 Cell Viability measurements

2.4.3.1 MTT Assay

MTT analysis was used to monitor the cell viability determined by the reduction of yellow MTT into purple formazan product by the dehydrogenases of metabolically active cells.

HepG2 cells were cultured in 96well plates in RPMI for 24h. After incubation the cells were then treated with concentrations of Atripla and incubated for a further 24h. Following the treatment period the media was then removed and 100µl of MTT (5 mg/mL) was added to each well and allowed to incubate for 45mins. After the incubation period the MTT solution was carefully removed and the purple crystals were solubilized with 100µl of DMSO, which was added to each well, and the plate was then read at the wavelength of 590nm with a microplate reader (ASYS). Finally the results were expressed as a percentage of absorbance observed in untreated cells (Mosmann, 1983).

2.4.4 Evaluation of cell death (Hoechst 33342/ propidium iodide staining)

Cell death has been characterized in all cell types and can be triggered in a programmed manner (apoptosis) in response to either extracellular or intracellular stimuli, or in a direct, uncontrolled mode, termed as necrosis. Cells were passaged and grown in 24 well plates with the density of 3×10^4 cells per well for 24h. The Cells were then treated with chosen drugs for 24h, the media used contained 1% FBS. The Cells were then incubated at 37°C containing 900 µg of 1% FBS containing media and 50 µg of both Hoechst and propidium iodide.

Hoechst 33342 and propidium iodide stained cells were visualised under an Axiovert 25 microscope with an excitation wavelength of 365 nm and emission of 420 nm. The cells counted in categories of live, apoptotic and necrotic cells were collected at randomly selected areas under 32 X magnification. Percentages of each category were obtained after dividing with the total cell count per well.

This method provides a semi-quantitative method for the evaluation of cell death. Cell counting is prone to observer bias and sensitive within a narrow limit of time

(approximately an hour after addition of the dyes) therefore each one was performed on different occasions to give N=3.

2.4.5 Oxidative Stress

2.4.5.1 Malondialdehyde assay (MDA)

Lipid peroxidation is the oxidative degradation of unsaturated lipids, which has a range of detrimental effects on the living cells. Malondialdehyde (MDA) is one of the products that result from lipid peroxidation, and its presence and quantity can be assayed, indicating oxidative degradation of lipids within the sample.

Thiobarbituric acid (TBA) forms a pink adduct with MDA. The degree of colour change indicates the level of MDA produced by the cells and this can be measured spectrophotometrically.

Malondialdehyde formation was utilized to quantify the lipid peroxidation in the liver, measured as thiobarbituric acid-reactive material.

HepG2 cells were prepared and plated in 12 well plates 24h prior to experiment. The media was then replaced with HIV drug containing media and incubated for another 24 hours.

After this period the media was removed and the cells washed with PBS. After the washing process the cells were centrifuged at 15000 rpm for 5 minutes. 150µl buffer was then added to each cell containing tube in order to lyse the cells (RIPA or TRIS HCl 0.1M and 1% SDS pH6.8, either with protease inhibitor mixed in a 25:1 ratio), and the sample mixture was then allowed to cool (2-8°C) for 30 minutes. The mixture was run through a 29G needle in order to break up the cells further before centrifugation in the cold room for a further 15 minutes.

125µL of the clear lysates were placed in glass tubes and to each test tube 50µL of 10% SDS (w/v), 250µL of 20% (v/v) acetic acid (pH 3) and 750µl of 0.67% (w/v) TBA was then added to each one. The solutions were then boiled at 100° for 1hour, and allowed to cool before 500µL distilled water was added. 80µL of each sample was taken and added to 96 well plates in duplicate and the absorbance measured at 532 nm. Results are expressed as a percentage of untreated cells.

2.4.5.2 nitroblue tetrazolium (NBT)

Cellular levels of oxidative stress were assessed by reduction of nitro blue tetrazolium (NBT).

HepG2 cells were grown in 12-well plates and incubated at 37 °C for approximately 48 hours. Following the incubation period the cells were then treated with different concentrations of the drug and 10 μmoles nitrotetrazolium blue (NBT) in treatment media and incubated for a further 4 hours. The media was then removed from the wells and replaced with 1 ml 70% ethanol to each well and left for 10 minutes to terminate the reaction.

The wells were then washed twice with 100% methanol to remove any excess NBT. Followed by air-drying of the wells at room temperature. The blue crystals were then solubilized in a 100 ml of a 6:5 mixture of 2M potassium hydroxide and DMSO (5:6). The solution from the different wells was transferred to individual wells in a 96 well plate and the absorbance was measured at 690 nm and results expressed as a percentage of absorbance in untreated cells.

2.4.5.3 Dichlorofluorescein (DCF)

The HepG2 cells were cultured in 96 well plates in RPMI for 24h. After incubation the cells were then treated with concentrations of efavirenz and incubated for a further 24h. Following the treatment period the media was then removed and 100 μl of 5 μM DCF in 0% FCS Media was added to each well and incubated for 30 mins. The cells were then washed with 100 μl of PBS and removed, 100 μL of PBS was added and the plate was then read at the excitation of 485nm and of emission is 528nm.

2.4.6 IL 8 Assay

Human IL8 ELISA set was obtained from BD OptEIA™ to measure IL8 release. Inflammation is determined by assessing the release of IL8 by hepG2 cells into the culture media following a step by step procedure from a human IL8 ELISA kit. The plate was then read at a wavelength 450nm with correction of 570nm.

2.4.7 ER Stress

2.4.7.1 Protein extraction sample preparation

After passaging the cells, 10mls of media containing cells were placed into round dishes and were left in the incubator at 37C for approximately a week. After a week the media was removed and add drug solutions were added to the cells and incubated for a further 24h.

After the incubation period the media was removed and cells were washed with 10mls PBS and removed. 5mls TBS was added and cells were scraped and transferred to 15ml tubes and centrifuged for 5mins. Media was removed and cells left at the bottom.

RIPPA buffer inhibitor cocktail (1ml RIPPA buffer, 40uls protease) was made and 150ul of inhibitor cocktail was added to samples and transferred to epindorph tubes and left in (5°C) for 30 mins. A syringe was used to lyse the cells and Centrifuged in the cold room at the highest RPM for 10 mins. The liquid was discard leaving the cells (pellet) and left in to freeze at -80°C in an upright position.

2.4.7.2 Western blot

Running gel and stacking gel was prepared as previously described and gel lanes were equiloaded. The samples were separated on a 7.5% polyacrylamide gel (SDS-PAGE) according to their molecular weight and Running' of samples at 100 V for approximately 15 minutes aided the entry of proteins into the running gel, into which they were allowed to 'run' for another 90 mins at 120V. After separation, the proteins were electrophoretically transferred to nitrocellulose membranes in a cooled Bio-Rad Transblot unit at 4 °C. The membranes were then incubated for an hour with 5% non-fat dry milk in Tween (50mls tween buffer and 2.5g milk powder) TBS in order to block the nonspecific binding sites of the proteins. The membranes were then incubated overnight at 4°C with wash buffer containing the primary antibodies CHOP-GADD 153 mouse monoclonal IgG 1:500 dilution) and Actin-B Actin mouse monoclonal IgG 1:25000 dilution.

The next day, the primary antibodies were removed and the membranes were washed 3 times (10 mins each) with wash buffer. Secondary antibody (goat antimouse IgG-HRP 30mls tween, milk powder and 6ul antibody) was then added in order to detect the bound primary antibodies and left to incubate at room temperature for 2hrs. Following incubation with the secondary antibodies, the membranes were washed for 30mins at 5mins intervals. Equal amounts of developing solutions were added to each films and left for 5 mins prior to development of the films.

2.5 Statistical analysis

Error bars shown in the various graphs represent means \pm standard error of the mean (SEM). All data shown are representative for at least two independent experiments. Statistical analyses were performed using GraphPad Prism 5 (GraphPad Software, Inc., La Jolla, CA, USA), with details described in the legends to the respective figures and appropriate statistical analysis. Differences between mean values for different groups were determined using the unpaired two-tailed Students t-test, differences between mean values within groups were evaluated using analysis of variance (ANOVA) with the appropriate post-test for comparison of multiple means. Statistical significance was indicated using * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$.

Chapter 3

The effects of HAART components on HepG2 cells, the mechanism of cell death.

3.1 Introduction

The development of HAART for HIV has greatly reduced the morbidity and mortality associated with patients developing AIDS. Notwithstanding the clinical efficiency of anti-retroviral drugs to suppress the HIV virus, the rising level of liver disease amongst HIV patients has become a cause of concern as it is not only an effect of the disease, but also side effects of the drugs.

Although the daily recommended dose for efavirenz is 600mg (3.17 to 12.67 μM detected in the plasma) some patients have shown to have approximately around C_{max} of 30-50 μM in their system which are similar to those used within this experiment (Apostolova et al., 2010a). C_{max} is the peak concentration that a drug achieves after it has been administered and before administration of a second dose.

The HepG2 cell line was derived from liver tissue with differentiated hepatocellular carcinoma and is frequently used to study the effects of drugs, liver injury and metabolism (Neuman et al., 1993) (Apostolova et al., 2012) because they retain biochemical characteristics and morphological properties of normal differentiated quiescent hepatocytes.

The aim of this chapter was to determine if components of Atripla cause damage to HepG2 cell line and to compare the cytotoxic effects of efavirenz with rilpivirine on the HepG2 cell line. Furthermore 2nd messenger involvement using inhibitors and direct measurement NF- κ B.

3.2 Methods

3.2.1 Experimental protocols

The MTT assay, described in the methods section was used in order to measure cell viability. HepG2 cells were treated with different concentrations of rilpivirine, efavirenz, emtricitabine and tenofovir (3,10,30,50,100 μM) or vehicle DMSO for 24h in 96 well plates. Concentration of NF- κ B inhibitors Bay 11-7082 (1,3,10 μM) and QNZ (25,50,100 μM) were also measured.

HPI staining was employed to evaluate cell death. Subconfluent levels of HepG2 cells in 24 well plates (40,000 cells/well) were treated with different concentrations of rilpivirine (1,3,10,30 μM) and efavirenz (3,10,30 μM), emtricitabine (3,10,30 μM) and tenofovir (3,10,30 μM) as described in the methods section.

The NBT assay was employed as described in methods to define if treatment of efavirenz (10,30,100 μM) leads to the production of superoxide in HepG2 cell line.

The intracellular DCF fluorescence was used as an index to quantify the overall Oxidative stress in HepG2 cell line. HepG2 cells were plated in 96 well plates and treated with efavirenz (10,30,100 μM) for 24 hours as explained in methods section.

The MDA assay was employed to determine if efavirenz leads to lipid peroxidation. HepG2 cells were plated in 12 well plates and treated with efavirenz (10,30,100 μM) for 24 hours as explained in methods section.

Human IL8 ELISA set was used to measure IL8 production. 96well plates containing the hepG2 cell line treated with different concentrations of rilpivirine (1,3,10,30 μM), efavirenz (3,10,30 μM), emtricitabine (3,10,30 μM) and tenofovir (3,10,30 μM) for 24h in 96 well plates. Bay 11-7082 (1,3 μM) and QNZ (25,50 μM) were also measured.

HepG2 cells were treated with different concentrations of efavirenz for 24h. The proteins were extracted and Western blot method was carried out to determine the presence of CHOP (ER stress marker), as explained in the previous chapter.

3.3 Results

3.3.1 The effect on HepG2 cell viability with exposure to emtricitabine, tenofovir, efavirenz and rilpivirine

Neither emtricitabine nor tenofovir causes a significant reduction in cell viability of HepG2 cells compared to control (Figure 3.1). They both cause a slight decrease in cell viability at 100 μ M however it is noteworthy that such high concentrations are unlikely to be achieved clinically.

Efavirenz proved to have the most hepatotoxic effect indicated by the decrease in cell viability of HepG2 cells with 30, 50 and 100 μ M (Figure 3.1). The hepatotoxic effects of efavirenz were found to be dose dependent with the loss of cell viability at 30, 50 and 100 μ M being significantly different when compared using one way ANOVA with Tukeys multiple comparison test to 3, 10 μ M.

A clear dose-responsive curve is seen with rilpivirine (Figure 3.2) with 10, 30 and 100 μ M there is a significant reduction in comparison to the control.

As can be seen from the data efavirenz and rilpivirine did not significantly reduce cell viability at low concentrations. Nevertheless, at the higher concentration of 30 μ M, efavirenz caused the most substantial decrease, decreasing the cell viability by 81% respectively while rilpivirine a 28% a decrease in the cell viability. It appears that efavirenz has more damaging effect than rilpivirine.

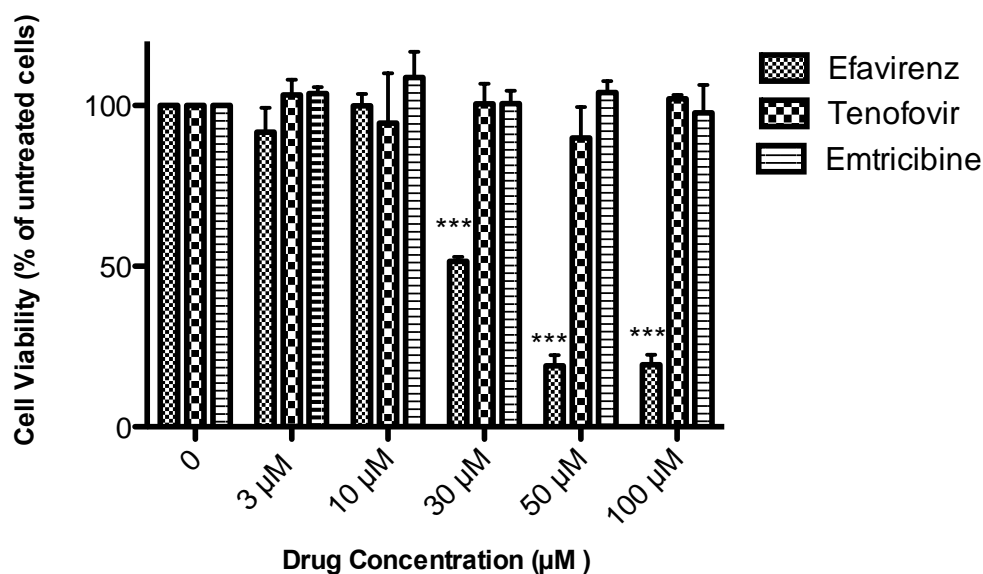


Figure 3.1. The effect of emtricitabine, tenofovir and efavirenz (3, 10, 30, 50 and 100µM) on the cell viability on the HepG2 cell line after 24 hours incubation. There was a dose dependent decrease seen with efavirenz and no difference seen with emtricitabine or tenofovir. Data represents mean % of untreated cells, \pm SEM n=3 (with 6 replicates per experiment). Statistical analysis was performed using One way ANOVA with Tukey's Multiple comparison test where $p < 0.05$ was considered significant; *** $p < 0.001$ compared to the untreated cells.

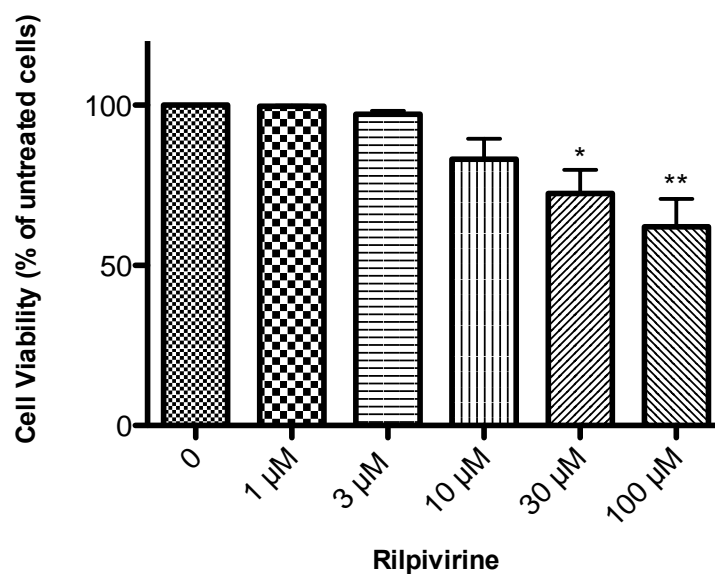


Figure 3.2. The effect of rilpivirine (1, 3, 10, 30, 50 and 100μM) on the cell viability (measured by MTT) on the HepG2 cell line after 24 hours incubation. A dose dependent decrease is observed. Data represents mean % of untreated cells, \pm SEM n=3 (with 6 replicates per experiment). Statistical analysis was performed using a One way ANOVA with Tukeys's multiple comparison test where $p < 0.05$ was considered significant; * $p < 0.05$ ** $p < 0.01$ *** $p < 0.001$ compared to the Control.

3.3.2 The effect of efavirenz on Oxidative Stress in HepG2 Cell line

3.3.2. 1 NBT, MDA and DCF assay

Oxidative stress was determined with using different methods. There was no detectable change in oxidative stress with efavirenz using NBT and MDA assays (table 3.1). However efavirenz (10, 30 μM) caused a significant increase in oxidative stress using the DCF assay (table 3.1).

efavirenz (μM)	NBT	MDA	DCF
0	100 \pm 1.8	100.4 \pm 4.4	100 \pm 1.5
10	105.3 \pm 4.1	116.9 \pm 13.1	124.5 \pm 3.8**
30	100 \pm 6.0	88.3 \pm 10.3	115.7 \pm 3.9**
100	71.9 \pm 10.7	84.6 \pm 18.7	106 \pm 11.2

Table 3.1 Results from the MDA, NBT and DCF assay carried out on subconfluent layers of HepG2 cells treated with different concentrations of efavirenz (10, 30 and 100 μM). Data represents mean % of untreated cells, \pm SEM n=3. Statistical analysis was performed using t-test where $p < 0.05$ was considered significant; * $p < 0.05$ ** $p < 0.01$ *** $p < 0.001$ compared to the untreated cells.

3.3.3 The mode of cell death on HepG2 cell line treated with emtricitabine, tenofovir, efavirenz and rilpivirine

A trend is seen with necrotic and apoptotic rates with 3 and 10 only those induced by 30 μ M efavirenz reached statistical significance after 24hr incubation period. There is a slight trend with emtricitabine and tenofovir however the data are not statistically significant (table 3.2). Rilpivirine also dose-dependently increased apoptosis and necrosis levels (table 3.3). Apoptosis and necrosis are a lot higher following rilpivirine exposure as compared to efavirenz, there is a two-fold increase in apoptosis and necrosis seen with 10 μ M efavirenz however, with rilpivirine there is a four-fold increase seen with apoptosis and a ten-fold increase in necrosis ($p < 0.05$ vs. efavirenz using Two way ANOVA with Bonferroni post test).

A

Drug Con (μM)	Efavirenz	Tenofovir	Emtricitabine
0	80.4 \pm 3.2	82.5 \pm 1.5	87.7 \pm 15.0
3	74.5 \pm 4.2	78.9 \pm 3.0	79.3 \pm 3.8
10	68.5 \pm 3.0 *	75.1 \pm 2.7	79.5 \pm 2.8
30	54.6 \pm 2.1 ***	73.6 \pm 3.0	76 \pm 2.8

B

Drug Con (μM)	Efavirenz	Tenofovir	Emtricitabine
0	3.7 \pm 0.9	4.8 \pm 1.4	2.3 \pm 0.5
3	6.0 \pm 1.3	5.8 \pm 0.8	4.9 \pm 1.1
10	6.4 \pm 0.8	6.5 \pm 1.2	5.9 \pm 1.1
30	10.1 \pm 1.3 **	7.3 \pm 1.3	6.0 \pm 1.0

C

Drug Con (μM)	Efavirenz	Tenofovir	Emtricitabine
0	15.9 \pm 2.9	13.7 \pm 0.8	12.0 \pm 2.8
3	19.4 \pm 3.7	15.3 \pm 2.7	15.9 \pm 2.8
10	25.1 \pm 3.1	18.3 \pm 2.3	14.7 \pm 1.8
30	35.3 \pm 1.5 **	19.1 \pm 2.6	17.2 \pm 2.1

Table 3.2 The effect of emtricitabine, tenofovir, efavirenz and on HepG2 cell death. The degree of live (A) apoptosis (B) and Necrosis (C) was found to be dose-dependently increased with efavirenz but was unchanged with tenofovir and emtricitabine. Data represents % of total cells \pm SEM n=3 (with at least 2 replicates per experiment). Statistical analysis was performed using Two Way ANOVA Two way ANOVA with Bonferroni post test multiple comparison test where each concentration was compared with the control. $p < 0.05$ was considered significant; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ compared to the untreated cells.

Rilpivirine (μM)	Live	Apoptotic	Necrotic
0	95.7 \pm 0.7	1.0 \pm 0.3	3.3 \pm 0.7
1	80.8 \pm 1.3 **	2.1 \pm 0.5	17.0 \pm 1.0**
3	75.9 \pm 3.0***	3.5 \pm 0.7**	20.5 \pm 2.8***
10	65.6 \pm 3.5***	4.1 \pm 0.4***	30.3 \pm 3.6***

Table 3.3 The effect of rilpivirine exposure on HepG2 cell death. Rilpivirine dose-dependently increased apoptosis and necrosis. Data represents % of total cells \pm SEM n=3 (with at least 2 replicates per experiment). Statistical analysis was performed using Two way ANOVA with Bonferroni post test where $p < 0.05$ was considered significant; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ compared to the untreated cells.

3.3.4 The effect of emtricitabine, tenofovir, Efavirenz, rilpivirine on HepG2 cell line IL-8 release

Figure 3.3 shows the IL-8 release by HepG2 cells after treatment with components of Atripla and Eviplera over a 24hr period. emtricitabine and tenofovir show no significant increase in IL-8 release. However 30 μM of efavirenz significantly increases IL-8 release compared to control.

Rilpivirine (Figure 3.4) appears to be different to efavirenz as a biphasic pattern is seen. At 3 and 10 μM there is a significant reduction in IL-8 and this is increased significantly at the higher concentration (30 μM), which suggests increased inflammatory response.

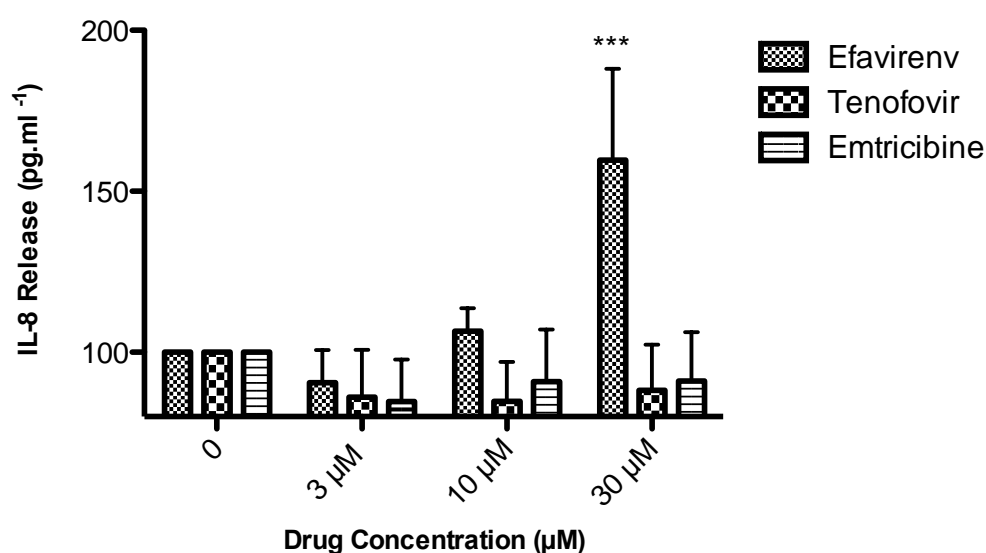


Figure 3.3. The effect of emtricitabine, tenofovir and efavirenz (3, 10 and 30 μM) on IL-8 release on the HepG2 cell line after 24 hours incubation. Emtricitabine and tenofovir show no significant increase in IL-8 production however with efavirenz significant amount of IL-8 production is seen with 30 μM compared to untreated cells. Data represents mean % of untreated cells \pm SE, n=3 (with at least 3 replicates per experiment). Statistical analysis was performed using one way ANOVA with Tukey's multiple comparison post test where $p < 0.05$ was considered significant; *** $p < 0.001$ compared to the untreated cells.

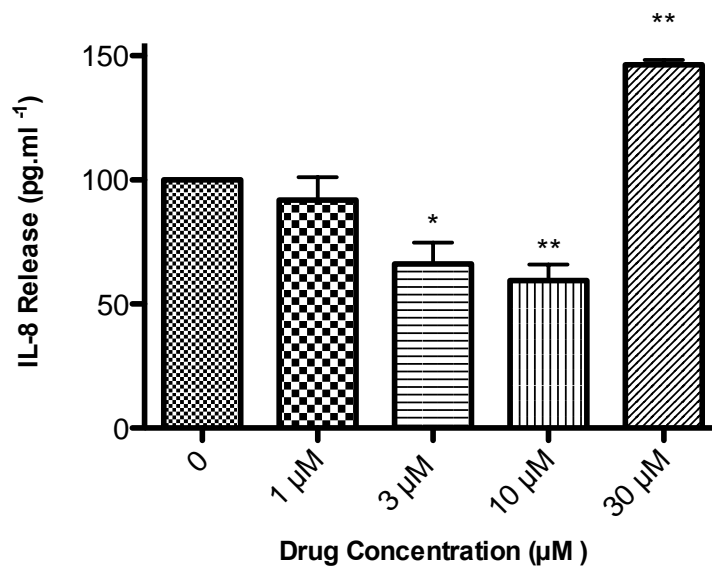


Figure 3.4 The effect of rilpivirine (1,3, 10 and 30µM) on IL-8 release on the HepG2 cell line after 24 hours incubation. A slight biphasic pattern is seen. Data represents mean % of untreated cells, \pm SEM n=3 (with at least 3 replicates per experiment). Statistical analysis was performed using one way ANOVA with Tukey's multiple comparison test where $p < 0.05$ was considered significant; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ compared to the untreated cells.

3.3.5 The effect of NF- κ B inhibition of efavirenz mediated HepG2 cell interleukin 8 release

BAY 11-7082 (BAY) is an inhibitor of κ B kinase (IKK) that has pharmacological activities that include anti-inflammatory effects. QNZ is also a potent NF- κ B inhibitor demonstrated to effect NF- κ B transcriptional activation and TNF- α production.

There is a significant reduction in cell viability with efavirenz 30 μ M when compared with the Control. When cultured HepG2 cells were incubated with efavirenz 30 μ M and the NF- κ B inhibitors there seems to be no protection with cell viability (Fig 3.5 A,C).

As the graph shows when Bay 11-7082 and QNZ are combined with efavirenz its IL-8 production is dramatically reduced. IL-8 release from liver cells have shown to be mediated by NF- κ B from previous studies (Wang et al., 2011) (Osawa et al., 2002). This data shows that it is a controlled release because its not effecting cell viability so much. Bay 11-7082 at 10 μ M shows to be back to control indicating it has blocked IL-8 release, there is protection afforded by co-incubation of efavirenz and NF- κ β inhibitors.

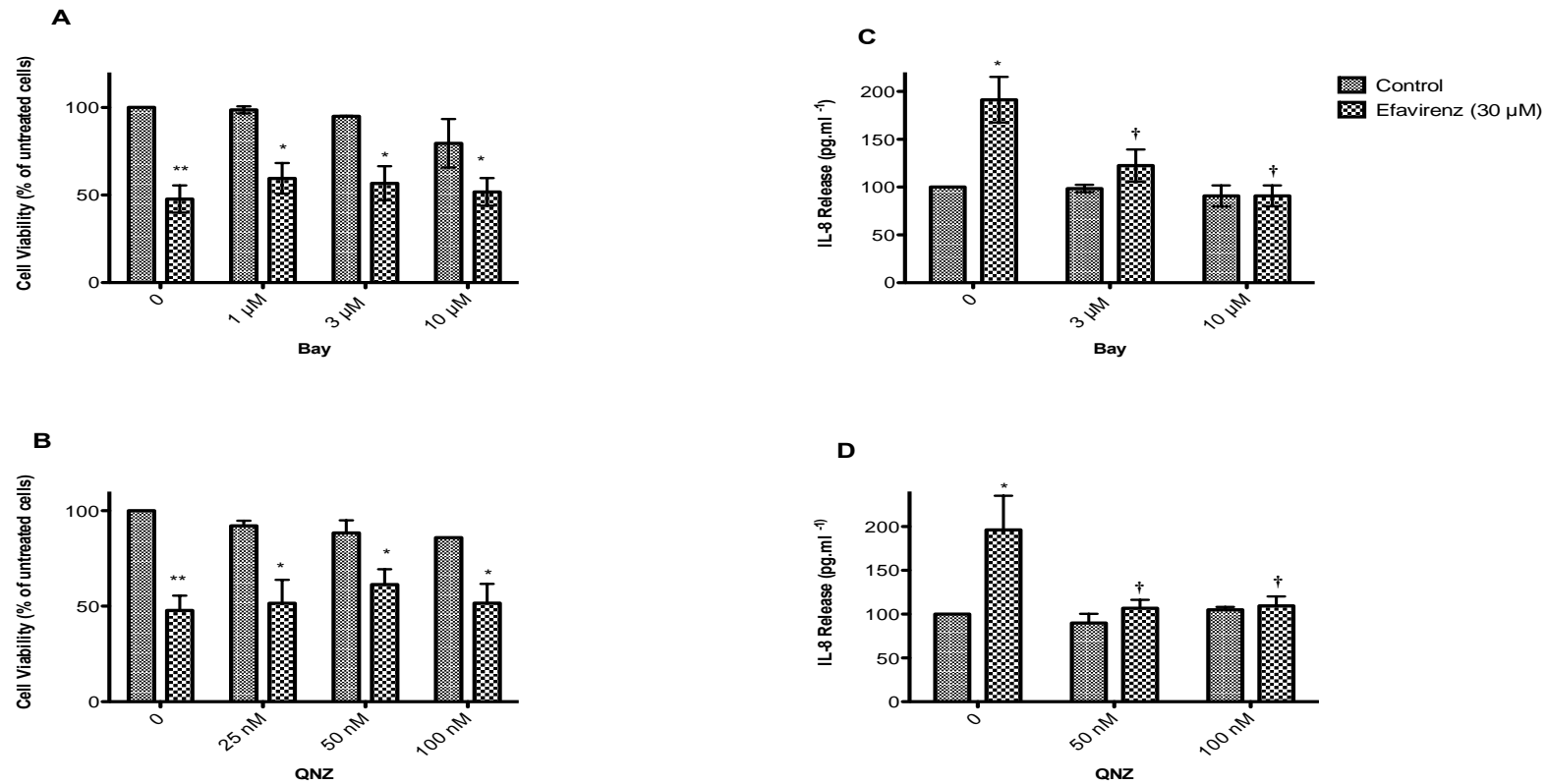
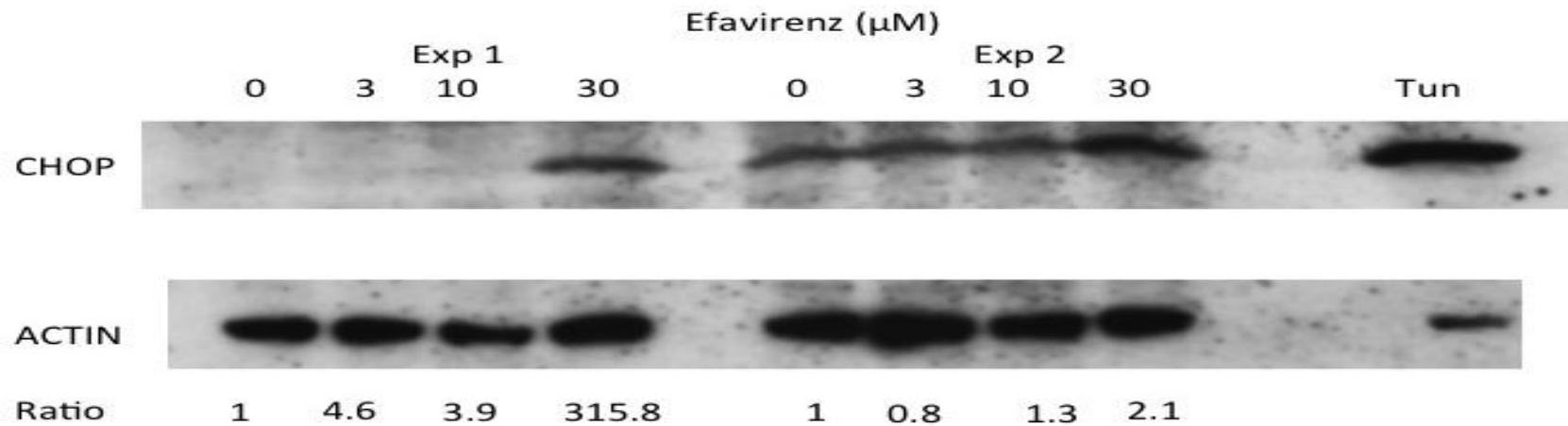


Figure 3.5 The effect of the NF- κ B inhibitor BAY 11-7085(1,3,10 μ M), QNZ (25,50,100nM) on efavirenz (30 μ M) mediated loss of cell viability (A,C) and IL-8 release (B,D) after 24 hours incubation. There is no protection seen with cell viability, however when Bay 11-7082 and QNZ are combined with efavirenz the IL-8 production is dramatically reduced therefore protection. Data represents % of total cells \pm SEM n=3 (with 3 replicates per experiment). Statistical analysis was performed using a two way ANOVA with Bonferroni post-test where $p < 0.05$ * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ compared to the untreated cells, † $p < 0.05$ compared to efavirenz alone.

3.3.6 Effect of efavirenz on ER Stress

Western blot was carried out in order to determine the role of ER stress in efavirenz mediated cytotoxicity carried out in HepG2 cells (Figure 3.6). The cells were treated with efavirenz, Tunicamycin and the membranes were blotted against ER stress marker CHOP.

Exposure to increasing concentrations of efavirenz (3,10, 30 μ M) showed to be dose dependant in increasing CHOP expression. Tunicamycin which is a well known ER stress inducer was used as a positive control and showed to produce a significant increase in CHOP levels when compared to the untreated cells.



Ratio: Chop/Actin

Figure 3.6 CHOP expression in HepG2 cells treated with efavirenz (3,10,30 μM) From both experiments carried out efavirenz 30 μM increases CHOP expression. CHOP expression levels were normalised to ACTIN expression using densitometry with expression of CHOP in untreated cells normalised to 1.

3.4 Discussion

For the first time, in this study the hepatotoxic effects of the components of Atripla and Eviplera were investigated on the on HepG2 cell line. The findings revealed that emtricitabine and tenofovir are unlikely to be responsible for hepatotoxic effects of Atripla and Eviplera because they had no effect on cell viability, cell death or inflammation even at concentrations that are not clinically relevant. However, the first and second-generation reverse transcriptase inhibitors efavirenz and rilpivirine both caused a loss of cell viability and an increase in apoptosis and necrosis. However, their effects on IL-8 release were different with Efavirenz causing dose dependent increase in IL-8, while rilpivirine had a biphasic effect decreasing IL-8 release at low concentrations while increasing release at higher concentrations.

Both efavirenz and rilpivirine caused loss of cell viability in a dose responsive manner, likely through increased cellular oxidative stress. Older NRTI's were linked to increased reactive oxygen species (ROS) production and consequent cellular oxidative stress, as NRTI's were able to inhibit DNA polymerase γ within the mitochondria, as well as inhibiting the HIV replication enzymes. As DNA polymerase γ within the mitochondria is inhibited, ROS are able to leak out of the electron transport chain and therefore induce oxidative stress. Efavirenz is known to cause mitochondrial dysfunction within the hepatocytes by inhibiting complex I of the electron transport chain, therefore disturbing the oxidative phosphorylation cycle that leads to increased ROS, reduced ATP synthesis, activate AMPK and consequently resulting in oxidative stress (Huyen et al., 2003). Apostolova et al. (Apostolova et al., 2010a) have shown that efavirenz directly affects the mitochondrial function in a reversible manner, decreases mitochondrial membrane potential, increasing mitochondrial superoxide production and reducing cellular glutathione content which suggests oxidative stress may play a role in efavirenz mediated toxicity. In addition to this Jamaluddin et al 2010 (Jamaluddin et al., 2010) have reported similar findings in human coronary artery endothelial cells with increased mitochondrial damage and oxidative stress. These reports strongly support the role of oxidative stress in efavirenz and rilpivirine-mediated cellular toxicity.

An increase in oxidative stress can damage cellular proteins, lipids, and DNA. Oxidative stress can cause direct damage to DNA leading to activation of DNA repair pathways. One of these pathways is via activation of poly (ADP-ribose) polymerase (PARP), which can become overactivated following increased oxidative stress with subsequent cell depletion of high-energy phosphates and NAD. PARP-mediated decrease in cellular ATP levels has been directly linked to cellular necrosis and this is known as the PARP suicide hypothesis. In addition overactivation of PARP has been linked to increased release from the mitochondria of apoptosis inducing factor (AIF) and cytochrome C both of which are linked to cellular apoptosis. The cell death data revealed a rise in both apoptotic and necrotic cell death in response to efavirenz and rilpivirine, this data might be explained by increased oxidative stress, DNA damage and overactivation of PARP resulting in both cellular necrosis and apoptosis. For future studies determining whether inhibitors of PARP can prevent the rise in necrosis and/or apoptosis in efavirenz and rilpivirine treated hepatocytes would confirm the involvement of this pathway.

Oxidative stress is also linked increased damage of proteins that can lead to ER stress. The secondary and tertiary protein structure can be disrupted by cellular oxidative stress resulting in increased ER stress as the cell attempts to correct the misfolded proteins. ER stress is characterised by an increased levels of incorrectly folded proteins in the ER lumen, which triggers UPR. This reaction then tries to reestablish the correct function of the ER, leading to enhanced ER protein folding capacity and increase protein degradation. NF κ B is activated with the increase in ER stress and oxidative stress leading to the activation of other cellular defense mechanisms and cell death.

Extensive damage to proteins can increase ER stress to such levels that the process can activate apoptotic pathways such as caspase 12 through increased expression of CHOP. Efavirenz exposure caused an increase in expression of CHOP in hepatocytes and this may explain the increased levels of cellular apoptosis observed. The increase in apoptosis with efavirenz confirms similar findings by Apostolova et al 2010 (Apostolova et al., 2010a). To date it is unknown whether rilpivirine also causes an increase in ER stress in hepatocytes, though rilpivirine has been shown to increase ER stress in cardiac myocytes (Mabley unpublished data). Therefore as it may be

possible that the increase in hepatocyte apoptosis following rilpivirine exposure may be mediated by ER stress, this would need to be confirmed with Western blotting experiments. The extent that increased ER stress plays in the increased hepatocyte apoptosis induced by both efavirenz or rilpivirine should be evaluated using chemical inhibitors of ER stress. Rilpivirine appears to be significantly more effective than efavirenz at increasing apoptosis and necrosis in hepatocytes and further work to determine why this is the case is required. Rilpivirine being a more potent reverse transcriptase inhibitor than efavirenz may have an increased damaging effect on the mitochondria and cellular oxidative stress levels or may have other cellular effects on proteins and DNA to activate necrosis and apoptosis pathways.

Interleukin 8 is a marker of inflammation and is produced in response to cellular stress or damage and it's often seen with HIV patients with liver disease such as NAFLD and NASH. The results from this study show a significant increase of IL-8 with higher concentration of efavirenz, which indicates cellular damage and inflammation. NF- κ B is a nuclear transcription factor and it is involved in regulating the expression of various cellular proteins that are involved in immune responses, inflammation, apoptosis, differentiation and proliferation. NF- κ B is activated when high levels of ROS are present. IL-8 is controlled and regulated by the activation of NF- κ B. There is a clear link with increased levels of ROS and efavirenz mediated toxicity, therefore this can be associated with increased IL-8 levels shown in this study. The IL-8 data revealed an increase in the IL-8 production and activation of NF κ B. The NF κ B was the proposed mechanism due to when NF κ B was inhibited, protection was seen and the IL-8 decreased dramatically back to the control. There was a dose dependent increase in IL-8 seen with efavirenz, however rilpivirine showed an interesting biphasic finding. At low concentrations of rilpivirine there was a decrease in basal IL-8 release, however at high concentrations of rilpivirine IL-8 release was increased. At low concentrations rilpivirine may be inhibiting NF κ B activation and reducing the transcription/translation of IL-8 protein reducing the basal release whereas at higher concentrations of rilpivirine NF κ B maybe being stimulated so increasing IL-8 release. Future work could reveal this mechanism by using inhibitors of NF κ B. Moreover IL-8 production is significantly increased with higher concentrations of efavirenz than rilpivirine.

Bay 11-7082 and QNZ have pharmacological activities that include anti-inflammatory effects. To confirm that the NF- κ B pathway was involved, HepG2 cells were treated with efavirenz in the presence of different inhibitors of NF- κ B along with appropriate controls. IL-8 production was reduced significantly in the presence of 10 μ m Bay 11-7082 and 50nM QNZ. A gradual reduction in the amount of IL-8 induced by HepG2 cell line was observed with increasing concentrations of Bay 11-7082 and QNZ. These results clearly suggest that the NF- κ B pathway might be involved in the induction of IL-8 by HepG2 cell line.

Emtricitabine and tenofovir belong to different classes of drugs to efavirenz. In addition both are drugs that require cellular phosphorylation to become active, tenofovir to a diphosphate form and emtricitabine to a triphosphate form. It is possible that the lack of effect of tenofovir and emtricitabine on hepatocyte cell viability may be because hepG2 cells have a reduced ability to phosphorylate these drugs to their active form (Dando and Wagstaff, 2004). Longer exposure times to tenofovir or emtricitabine to increase the time for phosphorylation may reveal as yet unseen hepatotoxic effects of these drugs. Both emtricitabine and tenofovir are nucleoside analogues designed to break the transcription of viral RNA to DNA prior to integration into the cells genome and not to interact with the reverse transcriptase enzyme. This suggests that emtricitabine and tenofovir are unlikely to interact and affect the activity of normal cellular proteins such as DNA polymerase- γ and mitochondrial complex I, both of which have been shown to previously be responsible for the increased oxidative stress and cellular damage observed with other retroviral drugs such as efavirenz.

Emtricitabine and tenofovir belong to different classes of drugs to efavirenz and rilpivirine and do not cause loss of cell viability or increase oxidative stress therefore may not affect the mitochondria and complex-1. The difference of activity, structure and mechanism of action might be the reason why they are not mitotoxic and therefore do not increase oxidative stress.

This study shows that efavirenz reduces cell viability and increases IL-8 production, both apoptotic and necrotic cell death through a rapid and concentration dependent manner, with 10µM inducing some changes and efavirenz 30 and 100µM proving to be extremely cytotoxic. Although an immobile cell system (*in vitro*) is clearly different from the *in vivo* situation, the experimental settings employed in this study aimed to resemble clinical conditions as close as possible hence the use of human hepatoma cell line HepG2. Moreover, the range of concentrations employed were chosen taking into account the important inter-individual variability reported in the pharmacokinetics of this drug.

This chapter identified that out of all the drugs from Atripla and Eviplera it is the efavirenz and rilpivirine components that cause damage to the mitochondria and cause hepatotoxicity. These drugs may not only be causing hepatotoxic effects directly, but may also be causing the patients on them to be more prone to developing other hepatic disease.

Chapter 4

**The effect of co-administration of efavirenz and palmitic acid on
HepG2 cells.**

4.1 Introduction

Recently the anti-retroviral drug efavirenz used to treat HIV positive patients to prevent them developing AIDS has shown to have adverse effects on the liver (Gao et al., 2010b, Ocama et al., 2010b, Apostolova et al., 2010b, Blas-Garcia et al., 2010) with 10% of patients treated with efavirenz demonstrating raised liver enzyme serum levels associated with hepatic toxicity and may require treatment cessation with this particular agent. These adverse effects of the liver have been shown to be enhanced by saturated fatty acids which themselves cause fatty liver disease (Gao et al., 2010a).

Fatty acids are important components of cell membranes and are vital for intracellular signalling as precursors of ligands that bind to nuclear receptors. Free fatty acids are vital energy stores however raised levels of circulating free fatty acids contribute to complications such as obesity and metabolic syndromes by promoting excess fat deposition in non adipose tissues that are unsuitable for fat storage such as in the liver. Fatty acids are chemically classified as saturated and unsaturated (monounsaturated and polyunsaturated) and their structure affects their biological effects. Palmitic acid, is the most common saturated fatty acid in the diet (Ricchi and et al., 2009).

Fatty liver disease is a growing problem in the western world as obesity levels increase. NAFLD is thus a hepatic manifestation of the metabolic syndrome. In NAFLD patients the decreased unsaturated fatty acids in ratio to raised saturated fatty acids in serum, adipose tissue and the liver is present that play a major role in the pathological syndromes.

FFA-induced programmed cell death is evident in many cell types, such as neuronal cells, human granulosa cells and hepatocytes (Ji et al., 2008). Many in vitro studies have shown a role for ER stress in saturated fatty acid- induced apoptosis, as seen in liver cells (Wei et al, 2006). Patients with NAFLD and NASH show to have significantly increased serum levels of IL-8. The exposure of hepatocytes to relevant pathophysiological concentrations (150 μ M is commonly associated with obesity (Laine et al., 2007)) of palmitic acid have shown to increase IL-8 levels (Joshi-Barve et al., 2003).

The aim of this chapter was to supplement the HepG2 cells with palmitic acid therefore mimicking the influx of excess FFAs to the hepatocytes and to investigate if efavirenz can enhance the damaging effects of palmitic acid on HepG2 cell line.

4.2 Methods

4.2.1 Experimental protocols

The MTT assay, described in the methods section was used in order to measure cell viability. HepG2 cells were treated with different concentrations of efavirenz (10, 30, 100 μ M) combined with palmitic acid (1, 3, 10 μ M) for 24h in 96 well plates.

HPI staining was employed to evaluate cell death as described in the methods section. HepG2 cells in 24 well plates were treated with different concentrations of efavirenz (1, 3, 10 μ M) combined with palmitic acid (10, 30, 100 μ M).

Human IL8 ELISA set was used to measure IL8 production. 96well plates containing the hepG2 cell line treated with different concentrations of efavirenz (1, 3, 10 μ M), combined with PA (3, 10, 30 μ M) for 24h.

4.3 Results

4.3.1 The effect on cell viability of HepG2 cells exposed to a combination efavirenz and Palmitic Acid.

There is a significant reduction in cell viability observed with efavirenz at 1 μ M plus 30 μ M of palmitic acid and 1 μ M plus 100 μ M palmitic acid. Also both 3 and 10 μ M show a significant reduction in cell viability with 10, 30 and 100 μ M of palmitic acid (Table 4.1). There is a dose response reduction in cell viability. It shows that even low concentrations of efavirenz when combined with palmitic acid have a significant reduction in cell viability showing cell toxicity when these two are combined. After palmitate exposure cell viability appeared to decrease with increasing levels of palmitic acid suggesting fatty acid decreased the rate of cell growth or increased cell death.

PA (μ M)	0	10	30	100
Efavirenz (μM)				
0	100 \pm 5.6	94.8 \pm 6.0	81.9 \pm 1.4	73.4 \pm 2.1
1	95 \pm 6.7	93.1 \pm 5.5	65.7 \pm 4.2**	46.7 \pm 11.4***
3	96.7 \pm 5.7	75.7 \pm 1.9***	61.4 \pm 1.5***	37.7 \pm 3.0***
10	95.6 \pm 4.8	69.3 \pm 3.7***	60.3 \pm 2.2***	36.5 \pm 3.5***

Table 4.1. The effect of efavirenz (1, 3, 10 μ M) on the cell viability in combination with palmitic acid (10, 30, 100 μ M) on the HepG2 cell line after 24 hours incubation. There is a dose dependent decrease in cell viability. Data represents mean % of untreated cells, \pm SEM n=3 (with 6 replicates per experiment). Statistical analysis was performed using Two way ANOVA with Bonferroni post test where p<0.05 was considered significant; **p<0.01, ***p<0.001 compared to the untreated cells.

4.3.2 The effect of palmitic acid and efavirenz on HepG2 cell death

Palmitic acid was clearly lipotoxic and induced a dramatic, dose- dependent increase in apoptotic and necrotic cell death in HepG2 cells (Table 4.2).

PA (μ M) Live	0	10	30	100
Efavirenz (μM)				
0	97.3 \pm 1.8	97.3 \pm 1.8	91.7 \pm 3.3	91.7 \pm 3.3
3	74.5 \pm 4.2**	84.9 \pm 0.4***	81.6 \pm 0.5*	55.0 \pm 4.1***
10	68.5 \pm 3.0**	79.1 \pm 1.2***	73.8 \pm 3.4**	46.2 \pm 2.7***

PA (μ M) Apoptotic	0	10	30	100
Efavirenz (μM)				
0	0.8 \pm 0.2	3.6 \pm 0.3	3.6 \pm 0.4	22.2 \pm 2.6***
3	6.0 \pm 1.3**	3.6 \pm 0.4**	5.6 \pm 0.9**	5.5 \pm 0.4
10	6.4 \pm 0.8**	5.8 \pm 0.5***	7.1 \pm 1.0***	6.7 \pm 1.8*

PA (μ M) Necrotic	0	10	30	100
Efavirenz(μM)				
0	1.9 \pm 1.6	4.3 \pm 3.2	5.0 \pm 2.9	5.0 \pm 1.7
3	19.4 \pm 3.7***	9.7 \pm 1.6**	8.9 \pm 1.0*	39.3 \pm 4.9***
10	25.1 \pm 3.1***	11.5 \pm 0.3***	13.9 \pm 0.7***	39.5 \pm 4.4***

Table 4.2 a, b, c The effect of efavirenz (3,10 μ M) and palmitic acid (0,30,100 μ M) on the HepG2 cell. Palmitic acid combined with efavirenz induced a dramatic, dose dependent increase in apoptotic and necrotic cell death in HepG2 cells. Data represents % of total cells \pm SEM n=3 (with at least 2 replicates per experiment). Statistical analysis was performed using Two Way ANOVA where each

concentration was compared with the control. $p < 0.05$ was considered significant; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ compared to the untreated cells.

4.3.3 The effect of the efavirenz and palmitic acid on HepG2 cell IL-8 release

These results demonstrate that HepG2 cells express large amounts of IL-8 in response to palmitic acid in a concentration-dependent manner. The IL-8 release is significantly higher as seen with efavirenz 3 μ M in combination with 10, 30 and 100 μ M palmitic acid (Table 4.3).

PA (μ M)	0	10	30	100
Efavirenz				
(μM)				
0	100 \pm 1.2	103.9 \pm 7.6	102.2 \pm 4.8	154.4 \pm 10.9*
1	115.4 \pm 5.2	144.8 \pm 30.2 \dagger	140.6 \pm 29.7 \dagger	147.9 \pm 46.4
3	111.5 \pm 10.0	197.2 \pm 25.2*** \dagger	184 \pm 30.1*** \dagger	175 \pm 35.0***

Table 4.3. The effect of efavirenz (1,3 μ M) and palmitic acid (10,30,100 μ M) on IL-8 release on the HepG2 cell line after 24 hours incubation. There is a dose dependent increase in the production of IL-8 with palmitic acid combined with efavirenz. Data represents mean % of untreated cells \pm SE, n=3 (with at least 3 replicates per experiment). Statistical analysis was performed using two way ANOVA with Bonferroni post test where $p < 0.05$ was considered significant; * $p < 0.05$ ** $p < 0.01$, *** $p < 0.001$ compared to the control $\dagger p < 0.05$ compared to palmitic acid alone.

4.4 Discussion

The build up of saturated fatty acids and their metabolites within the cells are known as lipotoxicity and have been associated with cellular injury and dysfunction. Lipotoxicity have been described previously in many cell types in different pathological conditions in cells such as the hepatocytes and have been shown to involve oxidative and ER stress. Both of these are involved in developing palmitic acid lipotoxicity leading to cell death however the involved pathways that are involved remain elusive (Weinberg, 2006, Guo et al., 2007, Wei et al., 2006).

Previous studies show that there is a “2-hit” hypothesis for the development and progression of NASH, first hit is the accumulation of fat in the liver which is susceptible to secondary insults that include inflammatory cytokines, that promote hepatocellular damage, inflammation, and progressive liver disease (McClain et al., 2004, Day, 2006). Increased supply of free fatty acids to the liver may play a major role in the development of hepatic inflammation (Joshi-Barve et al., 2007).

Palmitic acid is a saturated fatty acid which is linked to direct hepatic damage, it has also been proven that palmitic acid induces ER stress and apoptotic properties in hepatocytes and other cell lines. Palmitic acid may cause stress because of an increase in ROS, which then activates JNK pathway and inhibits the proapoptotic mediator bcl2/Bax, which modulates mitochondrial function leading to an increase oxidative stress. The data in this chapter demonstrate that pathophysiologically relevant concentrations of palmitic acid exposure to HepG2 cells increase IL-8 secretion and induce apoptotic and necrotic cell death.

For the first time this study showed the effect of efavirenz and palmitic acid on HepG2 cell line. efavirenz at a concentration of 10 μ M reduced cell viability down to 95.6%, palmitic acid at the concentration of 100 μ M reduced cell viability to 73.4% but in combination however reduced the cell viability to 36.5%. They are enhancing the damage when combined and this may be explained in 2 ways, firstly that there may be a synergistic damaging effect on the mitochondria increasing cellular

oxidative stress above that seen with either component alone or that efavirenz is increasing lipid uptake. As mentioned before efavirenz has been shown to cause lipid disturbances.

Palmitic acid was toxic to HepG2 cells in a concentration- dependent manner as it can be seen from the MTT assay. The reduction in cell viability was observed from palmitic acid concentrations of 10, 30, 100 μM of which 30 and 100 μM showed to be the most toxic to the cells, an increase in lipid uptake mediated by efavirenz may explain the increased loss of cell viability when both efavirenz and palmitic acid are combined. The combination of palmitic acid and efavirenz may also be increasing oxidative stress levels to very high levels and damaging cell viability. Palmitic acid can be utilised by the mitochondria to make ATP, efavirenz inhibits complex-1, palmitic acid tries to be metabolised and used for energy but complex-1 is inhibited by efavirenz therefore high production of oxidative stress.

The cell death assay showed apoptosis and necrosis by staining of nuclear morphology and membrane integrity. At a concentration of 100 μM palmitic acid produced a significant increase in both apoptotic and necrotic cell death. Moreover when palmitic acid was combined with efavirenz the number of cell death was significantly increased even at lower concentrations of palmitic acid. Past reports have exhibited similar findings with apoptosis and necrosis cell death pathways induced by palmitic acid by other authors on many cell types including hepatocyte cell line (Guo et al., 2007). Further studies have shown that palmitic acid induces ER stress and associated toxicity in many cell types including liver cells and preadipocytes demonstrated by western blotting (Wei et al., 2006, Guo et al., 2007).

The results show that the cells are sensitive to palmitic acid overload seen from the significant reduction in cell viability and raised levels of IL-8. Many number of mechanisms have been associated with palmitic acid induced apoptosis including ER stress and the activation of NF κ B, production of ROS which damages DNA, proteins, other macromolecules and lipids. It damages lipids through lipid peroxidation, which causes the fatty acids to become more hydrophilic and stiff which leads to an altered membrane structure resulting in damage, generation of oxidative stress (Beeharry et

al., 2003) altered mitochondrial permeability (Koshkin et al., 2008), changes in the morphology of the ER and an increase in ER stress (Cunha et al., 2008). The ability of palmitic acid to generate oxidative stress is well known, as is its ability to increase levels of markers of ER stress such as p-eIF2 α and CHOP (Cunha et al., 2008, Guo et al., 2007, Wei et al., 2006). Using these divergent pathways, palmitic acid is able to induce apoptosis via activation of protein kinase C, nuclear factor- κ B or JNK (Eitel et al., 2003). ER stress, which involves disruption of protein folding, lipid and sterol biosynthesis and intracellular calcium stores and ultimately cell death via apoptosis, ER stress has been associated with several pathological conditions, which can affect the liver.

Alone, both efavirenz and palmitic acid had similar effects on hepatocyte cell viability and IL-8 production, together, this study has provided evidence that the two work synergistically to enhance their effects, on cell viability, cell death and IL-8 production. Therefore efavirenz may make HIV patients more prone to NAFLD development. There are a number of possible mechanisms for why this synergy occurs. Firstly, together efavirenz and palmitic acid could induce an increased level of oxidative stress above the normal limits resulting in a build up of intracellular ROS. This would, in turn, result in the increase in IL-8 also recorded in this study. Further to this, elevated ROS could also result in increased activation of the NF- κ B and PA-1 pathways implicated in efavirenz and palmitic acid-mediated IL-8 production, explaining the synergistic elevation in IL-8 production.

Chapter 5

General Discussion

5.1 Main findings

HAART has changed the prognosis of AIDS. The development of such drugs was rapid and the focus was on clinical efficacy to reduce the mortality. However as the disease has become a controlled condition, there has been increasing emphasis placed on the long-term adverse effects induced by this life-long pharmacological treatment. There is a growing concern with side effects associated with efavirenz and the second-generation drug rilpivirine that includes hepatotoxicity. The association between plasma concentration and adverse hepatic effects is well established, and up to 10% of HIV patients treated with efavirenz present increased liver enzymes therefore discontinuation of the therapy.

The mitochondria is a major target for drug induced toxicity and a wide variety of mechanism such as oxidative stress and mitochondrial dysfunction are associated (Labbe et al., 2008). The objective of this study is to discuss the possible mechanisms underlying mitochondrial toxicity in relation to treatment with NNRTIs. Enhanced understanding of the mechanisms behind these effects is relevant for future management of long-term HAART and adverse effects in HIV-infected patients.

This investigation showed that the only components of Atripla and Eviplera that caused significant loss in the cell viability, an increase in the inflammatory marker (Interleukin 8 expression) and increased number of cell death on HepG2 cell line are efavirenz and rilpivirine. Efavirenz was shown to cause a dose-dependent loss of hepatocyte cell viability, increase in apoptosis and necrosis levels as well as increased inflammation as assessed by IL-8 release. Although rilpivirine is a second generation of NNRTI with an improved safety profile, it was also shown to cause loss of cell viability, and an increase in apoptosis and necrosis levels similar to that observed with efavirenz. However unlike efavirenz, rilpivirine had a biphasic effect on IL-8 release with low concentrations reducing basal release of IL-8 while higher concentrations increasing IL-8 release. It is likely that the deleterious effects of both efavirenz and rilpivirine are mediated through increased cellular oxidative stress. The increased levels of apoptosis may be via increased ER stress, which was observed in hepatocytes following efavirenz exposure.

Cell death assays confirmed efavirenz causes a significant dose dependent increase in apoptosis and necrosis. Rilpivirine shows the same dose dependent manner. It is likely that rilpivirine induces damage through a mechanism of which is similar to efavirenz, which involves mitochondrial dysfunction and increase oxidative stress.

Both efavirenz and rilpivirine carries a risk of hepatotoxicity and when combined with a diet high in fat it can lead to lipid accumulation in the liver, inflammation, cell injury and hepatic failure. Moreover efavirenz effects are partially mediated by activation of NF-kB. Furthermore fatty acids enhance damaging effects with efavirenz therefore patients treated with this medication have a higher risk of getting NAFL and liver failure. Patients on these drugs need to reduce the fat intake to reduce the risk of NAFLD and liver failure.

There is a strong link between obesity, increased circulating free fatty acids and liver steatosis. In patients with NAFLD there is increased lipolysis and increased delivery of FFAs to the liver. The higher the concentrations of free fatty acids the more severe the liver disease, therefore high levels of free fatty acids may be a contributing factor for the development and progression of NASH.

There is a significant rise in NAFLD and NASH associated with the western diet. NASH and NAFLD are known to increase IL-8 release and therefore clinically relevant in individuals with liver diseases on efavirenz and rilpivirine there these patients are more prone to developing liver disease (Apostolova et al., 2010a).

In conclusion, the present study demonstrates that both efavirenz and rilpivirine are mitotoxic and pro-apoptotic in human hepatic cells *in vitro*, which may be relevant to the understanding of the hepatotoxicity associated with these drugs. Moreover, since they are usually administered in combination with two NRTI, which are well known to have damaging effects on mitochondria, therefore potential drug combinations in which these and other serious side effects may be enhanced. Given that treatment is for life, these effects could easily accumulate and increase the liver toxicity induced. As seen from the results hepatocytes treated with efavirenz have a much larger damage profile than those treated with rilpivirine which coupled with the possible anti-inflammatory effect of rilpivirine at low concentrations suggests that Eviplera may be

a safer combination therapy than Atripla to treat HIV patients (Apostolova et al., 2010a).

5.2 Main limitations

This study aimed to elucidate some underlying mechanisms for the use of HAART drugs, time and cost made it challenging to expand some experiments. For example, there are a lack of mechanisms and experiments with rilprinine and palmitic acid. Further studies are required to give an underlying mechanism and confirm effects of rilpivirine. This will enable the answers to questions such as if rilpivirine causes ER stress, oxidative stress and the involvement of $\text{NF}\kappa\text{B}$ pathways.

The use of primary cells instead of cell lines could be advantageous. Furthermore the use of HIV infected cells could be beneficial in order to obtain more realistic findings into the effects of HAART.

5.3 Future perspective

Future studies may be useful to clearly ascertain the role of efavirenz and ER stress by western blot to highlight the activation of different pathways and the use of pharmacological inhibitors.

Involvement of ER stress and $\text{NF}\kappa\text{B}$ inhibitors and palmitic acid, oxidative stress involvement using NBT or DCF assays.

Moreover in vivo studies involving HIV infected animal models may be preferable to define a more of an understanding of long-term effects of HAART. The biphasic effect of rilpivirine on IL-8 could be investigated further and also if $\text{NF}\kappa\text{B}$ is involved.

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