Cardiovascular side effects of the antiretroviral agents rilpivirine, efavirenz, etravirine and abacavir: possible underlying mechanisms

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Abstract
The introduction of highly active antiretroviral therapy (HAART) has dramatically improved the life expectancy of HIV infected individuals. With the increased lifespan, complications associated with HAART are becoming more evident. Together with HIV itself and a higher prevalence of traditional risk factors, the antiretroviral agents are linked to an increase in cardiovascular diseases in the HIV population. In the present in vitro study the direct cardiac effects of a nucleoside reverse transcriptase inhibitor (NRTI) abacavir in combination with palmitic acid or cobalt chloride mimicking a diet high in saturated fatty acids or hypoxia respectively, were studied. Exposure to abacavir has been associated with an increased risk of myocardial infarction in clinical studies, but the damaging molecular mechanism remains elusive. Furthermore the in vitro cardiovascular effects of three non-nucleoside reverse transcriptase inhibitors (NNRTIs) were investigated; first generation NNRTI efavirenz and the second generation NNRTIs rilpivirine and etravirine.

In vitro experiments were performed on rat heart H9c2 cells or/and the human endothelial EA.hy926 cell line. The MTT assay was used to measure cell viability. Mode of cell death, either apoptotic or necrotic, was assessed by morphological analysis following propidium iodide/ Hoescht staining, measurements of histones in the cytoplasm and caspase activation. Endothelial function was evaluated by assessing acetylcholine-induced nitric oxide-mediated relaxation in male Sprague-Dawley rat aortic rings following exposure to rilpivirine or etravirine (1, 3 or 10 µM) for 2, 4 or 6h, and also with PJ34 (3µM), a PARP inhibitor, in combination with rilpivirine and etravirine (10µM) for 4 hours. The involvement of ER stress was assessed by measuring CHOP expression by Western blot and assessment of pro-inflammatory effects was evaluated by measuring IL-8 levels with an ELISA.

Treatment with abacavir on H9c2 cells showed no loss of cell viability or an increase in apoptosis or necrosis compared with controls. Combination with neither palmitic acid nor cobalt chloride altered the effects of abacavir. ER stress marker protein CHOP was not evident in proteins extracted from H9c2 cells treated with abacavir. A dose dependent increase in CHOP expression was seen in response to both cobalt chloride and palmitic acid treatments, however abacavir did not enhance this response. H9c2 cells exposed to the NNRTIs (efavirenz, rilpivirine and etravirine) showed decreased cell viability and increased cell apoptosis and necrosis. All three NNRTIs increased cellular expression of CHOP indicating ER stress may mediate the loss of cell viability. Caspase activation was seen with all three NNRTIs. Similar results were obtained with endothelial EA.hy926 cells. This was however in the absence of ER stress. All NNRTIs stimulated an increase in IL-8 expression compared with controls, showing a pro-inflammatory effect. Etravirine induced a tenfold increase of IL-8 than rilpivirine and efavirenz. Both rilpivirine and etravirine were associated with PARP-mediated endothelial dysfunction, but to a lesser extent than efavirenz.

An underlying pathogenic mechanism explaining the possible increased risk of myocardial infarction following abacavir exposure was not found in the performed experiments. Further studies are necessary to determine the possible damaging effects of abacavir on the cardiovascular system. All NNRTIs caused cardiovascular cell damage, possibly through different mechanisms in cardiac and endothelial cells. The cardiac cell damage may be mediated through increased ER stress, whereas endothelial cell damage and dysfunction may partially be mediated through PARP and the pro-inflammatory effects of the NNRTIs. These results warrant further cardiovascular studies and monitoring in patients on long term NNRTI treatment.
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Abbreviations

ABC  abacavir
ACh  acetylcholine
ADP  adenosine diphosphate
AIDS acquired immunodeficiency syndrome
ART  antiretroviral therapy
ATP  adenosine triphosphate
BHIVA British HIV association
BH4  tetrahydrobiopterin
CCR5 chemokine receptor type 5
CD4  cluster of differentiation 4
cGMP cyclic guanosine monophosphate
CHD  chronic heart disease
CHOP CEBP homologous protein
cNOS constitutive nitric oxide synthase
COCl₂ cobalt chloride
CVA  cerebrovascular accident
CVD  cardiovascular disease
CXCR4 chemokine receptor type 4
D:A:D The Data Collection on Adverse events of Anti-HIV Drugs
DMEM Dulbecco's Modified Eagle Medium
DMSO dimethyl sulfoxide
<table>
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<td>DNA</td>
<td>deoxyribonuclease</td>
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<td>efavirenz</td>
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<td>eNOS</td>
<td>endothelial nitric oxide synthase</td>
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<td>ER</td>
<td>endoplasmic reticulum</td>
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<td>etravirine</td>
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<td>FAD</td>
<td>flavin adenine dinucleotide</td>
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<td>FBS</td>
<td>fetal bovine serum</td>
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<tr>
<td>FMD</td>
<td>flow mediated dilation</td>
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<tr>
<td>FMN</td>
<td>flavin mononucleotide</td>
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<tr>
<td>gp 41</td>
<td>glycoprotein 41</td>
</tr>
<tr>
<td>gp120</td>
<td>glycoprotein 120</td>
</tr>
<tr>
<td>HAART</td>
<td>highly active anitretroviral therapy</td>
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<tr>
<td>HDL</td>
<td>high density lipoprotein</td>
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<tr>
<td>HIV</td>
<td>human immunodeficiency virus</td>
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<tr>
<td>HPI</td>
<td>Hoescht/Propidium iodide</td>
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<td>inducible nitric oxide synthase</td>
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<td>MDA</td>
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<tr>
<td>mRNA</td>
<td>messanger ribonucleic acid</td>
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<tr>
<td>MTT</td>
<td>3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide</td>
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<tr>
<td>NAD+</td>
<td>nicotinamide adenine dinucleotide</td>
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<tr>
<td>NADPH</td>
<td>nicotinamide adenine dinucleotide phosphate</td>
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<td>NBT</td>
<td>Nitrotetrazolium blue (nitrobluetetrazolium)</td>
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<tr>
<td>NRTI</td>
<td>nucleotide reverse transcriptase inhibitors</td>
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<tr>
<td>nNOS</td>
<td>neuronal nitric oxide synthase</td>
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<td>non-nucleoside reverse transcriptase inhibitors</td>
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<td>nucleotide reverse transcriptase inhibitor</td>
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<tr>
<td>O$_2^-$</td>
<td>superoxide</td>
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<td>poly (ADP-ribose) polymerase</td>
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<td>phosphate buffered saline</td>
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<td>PE</td>
<td>phenylephrine</td>
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<tr>
<td>PI</td>
<td>protease inhibitors</td>
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<td>ribonucleic acid</td>
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<td>reactive oxygen species</td>
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<td>rilpivirine</td>
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<tr>
<td>SDS</td>
<td>sodium dodecyl sulphate</td>
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<td>SEM</td>
<td>standard error of the mean</td>
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<tr>
<td>SOD</td>
<td>superoxide dismutase</td>
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<td>sarcoplasmic reticulum</td>
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<td>T</td>
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<td>tumour necrosis factor-alpha</td>
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<tr>
<td>UPR</td>
<td>uncoupled protein response</td>
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<tr>
<td>WHO</td>
<td>World Health Organisation</td>
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Dedication

This thesis is dedicated to my parents Herdis and Ola and Sue and Artie Wearing.
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To my fantastic friends, none mention but none forgotten, thank you for putting up with my rants, and thank you for many celebrations.

Lastly, but not least, biggest thanks to my favourite four-legged friend Jack and the weather gods for providing me with many days with beautiful, strong south-westerly winds, both which have helped me keep my sanity over the last four years.
Authors Declaration

I declare that the research contained in this thesis, unless otherwise formally indicated within the text, is the original work of the author. The thesis has not been previously submitted to this or any other university for a degree, and does not incorporate any material already submitted for a degree.
Chapter One: General introduction
1.1 History and epidemiology of HIV and AIDS

Acquired Immune Deficiency Syndrome (AIDS) was officially recognised in 1981 and the causative agent, human immunodeficiency virus (HIV) identified two years later. The World Health Organisation (WHO) estimated that 35.3 million people were living with HIV worldwide at the end of 2012. In the UK, 98,400 individuals were estimated to be living with HIV in 2012 by the The Health Protection Agency (HPA). There is an increasing number of HIV patients being diagnosed with HIV over the age of 50 (Grabar, Weiss et al. 2006). The HPA reports roughly a doubling in diagnoses in people aged ≥50 years in 2012 in comparison to 2003 in the UK. In 2012, 1 in 4 people accessing HIV care were over 50 years (figure 1.1).

Figure 1.1  Age spread in the current HIV population. Obtained from the Health Protection Agency (2012).
1.2 The structure and lifecycle of HIV

HIV consists of an outer lipid membrane (envelope) with proteins gp120 and gp41 protruding from its surface. Two identical RNA strands and several viral proteins: reverse transcriptase, integrase and proteases are contained within a capsid made from p24 within the viral envelope (figure 1.2).

Figure 1.2  The structure of human immunodeficiency virus. HIV is spherical in shape and consists of the viral RNA contained in a capsid (Clark 2009).

The lifecycle of HIV is well characterised (Volberding 2008). HIV enters human cells in three phases. Initially the HIV envelope protein gp120 binds to CD4 receptors located in lipid rich areas within the cell membrane of the target cells. This leads to a conformational change in the viral envelope, which allows the binding of the virion
to a co-receptor, either CCR5 or CXCR4 on the target cell. This in turn leads to a further conformational change in one of the viral envelope proteins, gp41, which brings about fusion of the virion with the cell membrane and entry of the HIV into the cell cytoplasm. Once the virus has reached the cytoplasm, the next stage is poorly understood and involves removal of the viral protein coat. This releases a range of components, among others, the enzyme reverse transcriptase which copies the single stranded viral RNA into double stranded DNA. The viral DNA crosses the nuclear membrane. HIV integrase mediates integration of the viral DNA into the host DNA. The resulting provirus might be latent or it might be actively transcribed into mRNA. The latter leads to the production of a range of viral proteins (Nef, Tat and Rev amongst others), each with a specific function supporting viral replication and the formation of new HIV virions. The newly assembled virions bud from the host cell and are ready to infect new cells (figure 1.3).
Theus Integration Function
The Reverse Onion Replica
The US lifecycle

Figure 1.3 HIV infecting a human cell/HIV lifecycle (Clark 2009).
1.3 The immune system and HIV

The HIV infects cells of the immune system, the main target being CD4 T-lymphocytes. This leads to destruction of and therefore a decline in the number of CD4 T-lymphocytes. In uninfected individuals, the CD4 T-lymphocytes count is between 450-1600 CD4 cells/mm³. Although the rate of disease progression varies, without pharmacological intervention the immune system in the majority of HIV infected individuals fails to control viraemia, and they ultimately progress to develop AIDS and succumb to infectious organisms.

1.4 Antiretroviral therapy

The ideal solution to the HIV pandemic is a vaccine delivered to those at risk. So far, the research in this area has been largely unsuccessful. Until a successful vaccine candidate becomes available, treatment to improve the life expectancy and quality of life of those affected is of paramount importance. Treatment does not eliminate the virus, but keeps the infection under control and is life-long.

The aims of antiretroviral treatment are to suppress viral replication to undetectable levels (< 50 viral RNA/ml) and to restore CD4 T-lymphocytes levels to near normal levels. In most patients this is achievable 12 weeks after initiation of antiretroviral treatment (Lepri, Miller et al. 2001).
1.4.1 **Antiretroviral drugs used in the treatment of HIV**

Antiretroviral agents used in the lifelong treatment of HIV act at different stages in the HIV replication cycle and are classified thereafter (figure 1.4 and table 1.1).

1.4.1.1 Entry and fusion inhibitors

Entry and fusion inhibitors act early in the HIV life cycle and prevent the virus from entering the host cells. Currently two agents are on the UK market: maraviroc and enfuviritide (BNF 2014). Enfuviritide is a fusion inhibitor and works by interacting with gp41, which renders the virus incapable of fusing with the cell membrane (De Clercq 2009). The downside to enfuviritide is that it has a peptide-based structure, and as such requires subcutaneous administration, which limits its usability. Maraviroc is a CCR5 antagonist and therefore prevents the viral interaction with the co-receptor, which is necessary for HIV entry into the host cell. The downside to this drug is that it is only active against a particular strain of the virus, R5, which will favour the selection of X4 strains which can still enter the cells (De Clercq 2009).

1.4.1.2 Nucleoside and nucleotide reverse transcriptase inhibitors (NRTIs/ NtRTIs)

The antiviral era started with the discovery of zidovudine (AZT), an NRTI, in 1985 (Mitsuya, Weinhold et al. 1985). The FDA approved AZT for the treatment of HIV and AIDS in 1987 and several NRTIs followed. These agents are all pro-drugs that structurally resemble nucleosides/ nucleotides. Intracellular phosphorylation is
necessary for the drugs to exert their antiviral properties. The phosphorylated derivatives compete with the nucleotides and nucleosides produced by the host cells and are incorporated into the viral DNA chain. As these compounds are lacking a 3’hydroxyl group, incorporation leads to chain termination (De Clercq 2009). There are currently 6 NRTIs on the market in the UK (BNF 2014), 5 of which are nucleoside transcriptase inhibitors: abacavir, lamivudine, stavudine, emtricitabine, didanosine and zidovudine, and one nucleotide transcriptase inhibitor: tenofovir.

1.4.1.3 Non nucleoside reverse transcriptase inhibitors (NNRTIs)

NNRTIs are chemically distinct from NRTIs and NtRTIs and do not require internal phosphorylation for their activation. The mode by which they inhibit HIV reverse transcriptase is also different: they bind to an allosteric site on the viral enzyme, which leads to a conformational change within the enzyme. This in turn stops synthesis of viral DNA (Reynolds, de Koning et al. 2012). The first generation NNRTI nevirapine was followed by the approval of efavirenz and more recently the second-generation drugs: etravirine and rilpivirine.

1.4.1.4 HIV integrase inhibitors

Integrating the viral DNA into the host DNA is paramount for the HIV replication cycle. This is a multistep process in which HIV integrase is involved in two of the steps; firstly processing the viral DNA through cutting and joining reactions and secondly covalently linking the viral DNA to the host DNA, termed DNA strand
transfer (Hazuda, Felock et al. 2000). Raltegravir was approved for the treatment of HIV in 2007 and exerts its antiviral action by preventing the strand transfer step (Temesgen and Siraj 2008). Recently a second HIV integrase inhibitor has been approved for the treatment of HIV: dolutegravir.

1.4.1.5 HIV protease inhibitors (PIs)

HIV protease cleaves peptide bonds in the viral proteins produced by the host cell. HIV protease inhibitors bind to HIV protease, and prevent the maturation of the proteins leading to viral particles with a reduced ability to infect new host cells. Several drugs in this class are currently on the market: atazanavir, ritonavir, indinavir, darunavir, fosamprenavir, nelfinavir, saquinavir and tipranavir. Although ritonavir is a protease inhibitor, in clinical practice it is used as a pharmacokinetic booster in low doses due its ability to inhibit CYP3A4, an enzyme that metabolises other HIV drugs. All the protease inhibitors except nelfinavir are boosted with low-dose ritonavir.
Figure 1.4  Mode of action of different classes of antiretroviral drugs (Clark 2009)
<table>
<thead>
<tr>
<th>PI</th>
<th>NRTIs/NtRTIs</th>
<th>NNRTIs</th>
<th>Entry and fusion inhibitors</th>
<th>Integrase inhibitors</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ritonavir</td>
<td>Abacavir (g)</td>
<td>Efavirenz</td>
<td>Enfuvirtide</td>
<td>Raltegravir</td>
</tr>
<tr>
<td>Lopinovir</td>
<td>Didanosine (a)</td>
<td>Rilpivirine</td>
<td>Maraviroc</td>
<td>Dolutegravir</td>
</tr>
<tr>
<td>Fosamprenavir</td>
<td>Tenofovir (a)</td>
<td>Etravirine</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Darunavir</td>
<td>Emtricitabine (c)</td>
<td>Nevirapine</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Saquinavir</td>
<td>Stavudine (t)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Indinavir</td>
<td>Zidovudine (t)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Atazanavir</td>
<td>Lamuvidine (c)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tipranavir</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 1.1  Different antiretroviral drugs and their therapeutic class (BNF 2014). 
(t) = thymidine analogue, (a) = adenosine analogue, (g) = guanosine analogue, (c) = cytidine analogue.

### 1.4.2 Treatment guidelines and treatment choice

The British HIV Association (BHIVA) guidelines recommend starting antiretroviral treatment when the CD4 counts ≤ 350 cells/mm³ with a backbone of 2 NRTIs/NtRTI in combination with an NNRTI, integrase inhibitor or ritonavir boosted PI (table 1.2). The combination of three or more agents is commonly referred to as highly active antiretroviral therapy, HAART. Beyond the primary aim of keeping viraemia low, preventing drug resistance from developing is of paramount importance. The choice of ART regime is made more challenging by the presence of co-morbidities,
polypharmacy and drug interactions, pregnancy, overlapping toxicity of different antiretroviral agents, adverse effects, cost, availability and drug resistance.

<table>
<thead>
<tr>
<th>First line</th>
<th>Alternative</th>
</tr>
</thead>
<tbody>
<tr>
<td>NRTI</td>
<td></td>
</tr>
<tr>
<td>Tenofovir and emtricitabine</td>
<td>Abacavir and lamuvudine</td>
</tr>
<tr>
<td>EFV</td>
<td></td>
</tr>
<tr>
<td>Nevirapine or rilpivirine</td>
<td></td>
</tr>
<tr>
<td>Atazanavir and ritonavir</td>
<td>Lopinavir with ritonavir</td>
</tr>
<tr>
<td>Darunavir and ritonavir</td>
<td>Or fosamprenavir with ritonavir</td>
</tr>
<tr>
<td>Integrase inhibitor</td>
<td></td>
</tr>
<tr>
<td>Raltegravir</td>
<td></td>
</tr>
</tbody>
</table>

Table 1.2 First and second line treatment in HIV patients initiated on ART in the UK. BHIVA recommends a combination of two NRTIs/NtRTI with either an NNRTI, PI or integrase inhibitor.

1.5 Adverse effects of antiretroviral therapy

Treatment with antiretroviral therapy is lifelong and side effects are common. Some side effects are class specific whereas others are specific to a particular drug. A review by Esser et al. in 2007 summarised the side effects of HIV therapy (table 1.3).
<table>
<thead>
<tr>
<th>Drug or drug class</th>
<th>Commonly reported side effects</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reverse transcriptase inhibitors</td>
<td></td>
</tr>
<tr>
<td>Abacavir</td>
<td>Hypersensitivity syndrome</td>
</tr>
<tr>
<td>Didanosine</td>
<td>Pancreatitis, neuropathy, lipoatrophy</td>
</tr>
<tr>
<td>Emtricitabine</td>
<td>Headache</td>
</tr>
<tr>
<td>Lamivudine</td>
<td>Headache</td>
</tr>
<tr>
<td>Tenofovir</td>
<td>Rarely affects renal function, hypophosphatemia</td>
</tr>
<tr>
<td>Stavudine</td>
<td>Neuropathy, pancreatitis, lipoatrophy</td>
</tr>
<tr>
<td>Zidovudine</td>
<td>Neutropenia, anaemia, myopathy, lipoatrophy</td>
</tr>
<tr>
<td>Protease inhibitors</td>
<td></td>
</tr>
<tr>
<td>Class effect</td>
<td>Glucose intolerance, disturbances of lipid metabolism, gastrointestinal upset, lipodystrophy syndrome</td>
</tr>
<tr>
<td>Atazanavir</td>
<td>Hyperbilirubinemia, diarrhoea, headaches</td>
</tr>
<tr>
<td>Darunavir</td>
<td>Diarrhoea, nausea, hyperlipidaemia</td>
</tr>
<tr>
<td>Fosamprenavir</td>
<td>Diarrhoea, hyperlipidaemia</td>
</tr>
<tr>
<td>Indinavir</td>
<td>Nephrolithiasis, hyperbilirubinemia, dry skin and mucous membranes, hyperlipidaemia</td>
</tr>
<tr>
<td>Nelfinavir</td>
<td>Diarrhoea, nausea</td>
</tr>
<tr>
<td>Ritonavir</td>
<td>Diarrhoea, nausea, perioral dysesthesia, hepatotoxicity, hyperlipidaemia (dose dependent)</td>
</tr>
<tr>
<td>Saquinavir</td>
<td>Diarrhoea, nausea (usually mild), hyperlipidaemia</td>
</tr>
<tr>
<td>Tipranavir</td>
<td>Diarrhoea, nausea, hepatic toxicity, hyperlipidaemia</td>
</tr>
<tr>
<td>Class effect</td>
<td>Cutaneous drug reaction</td>
</tr>
<tr>
<td>Non-nucleoside transcriptase inhibitors</td>
<td></td>
</tr>
<tr>
<td>Efavirenz</td>
<td>Psychotropic side effects</td>
</tr>
<tr>
<td>Etravirine</td>
<td>Rash</td>
</tr>
<tr>
<td>Nevirapine</td>
<td>Hepatotoxicity</td>
</tr>
<tr>
<td>Rilpivirine</td>
<td></td>
</tr>
<tr>
<td>Entry and fusion inhibitors</td>
<td></td>
</tr>
<tr>
<td>Enfuvirtide</td>
<td>Cutaneous drug reaction, local reaction at injection site</td>
</tr>
<tr>
<td>Maraviroc</td>
<td></td>
</tr>
<tr>
<td>Integrase inhibitors</td>
<td></td>
</tr>
<tr>
<td>Raltegravir</td>
<td></td>
</tr>
<tr>
<td>Dolzugravir</td>
<td></td>
</tr>
</tbody>
</table>

Table 1.3  Commonly reported side effects of ART. The table is adapted from “Side effects of HIV therapy” by Esser et al. 2007 (Esser, Helbig et al. 2007).
More recently, the increased prevalence of cardiovascular diseases in the HIV population has lead to an increase in research efforts investigating the effects of antiretroviral treatment on the myocardium and vasculature (Dube, Lipshultz et al. 2008, Landay, Bavinger et al. 2013).

1.6 Cardiovascular system

1.6.1 Anatomy and physiology of the cardiovascular system

The cardiovascular system consists of the heart, the blood vessels and the blood. The anatomy and physiology have been well characterised (Patton and Thibodeau 2010, Aaronson, Ward et al. 2013).

The heart is made up of a right and left atrium connected to a corresponding ventricle via the tricuspid and mitral atrioventricular valve, respectively. Three structurally different tissue layers make up the heart: the epicardium, myocardium and the endocardium. The epicardium is the outermost layer of the heart and the endocardium the innermost layer lining the inside of the chambers and the heart valves. The bulk of the heart is termed the myocardium, and consists of myocardial cells that contract rhythmically and continuously, and maintain blood flow through the blood vessels. The myocardial cells are connected together via intercalated discs, and ensures the myocardium acts as a single functional unit. The myocardial cells are rich in mitochondria in order to meet the energy demands of the contractile function. ATP and calcium are necessary for contraction. Calcium enters the
myocytes through calcium channels from the extracellular environment and from
the sarcoplasmic reticulum (SR).

The blood vessels are divided into three main types: arteries, veins and capillaries. The arteries carry blood away from the heart, the veins carry blood towards the heart whilst the capillaries are the microscopic vessels that carry blood from small arteries to small veins (from arterioles to venules). The larger blood vessels have a tri-laminar structure. Tunica adventitia is the outermost layer and consists of collagen fibrils, nerve endings, blood supply, fibroblasts and mast cells. The tunica media is between the tunica intima and the adventitia, and consists of smooth muscle cells and extracellular matrix, of which elastin is abundant. The tunica intima is the innermost layer and consists of a thin monolayer of endothelial cells on a bed of extracellular matrix molecules known as the basement membrane. The composition of the extracellular matrix molecules changes with age: non-fibrillar collagens are replaced by fibrillar forms with age, together with an increase in smooth muscle cells leading to thickening of the blood vessel wall.

The endothelium has many important functions, which include acting as a barrier between the vessel lumen and the underlying tissues thereby controlling the movement of substances, involvement in angiogenesis, inflammation, thrombosis and fibrinolysis, as well as controlling vascular tone.
1.6.2 The role of nitric oxide (NO) and endothelin-1

Vascular tone is maintained by secreting substances that either lead to contraction or relaxation of the underlying vascular smooth muscle. Important vasodilators include nitric oxide (NO), prostacyclin and endothelium-derived hyperpolarizing factor. Important vasoconstrictors include endothelin-1, thromboxane A<sub>2</sub> and prostaglandin H<sub>2</sub> (Aaronson, Ward et al. 2013).

Nitric oxide, the main vasodilator released from endothelial cells, is produced from the amino acid L-arginine via a two-step process, which is catalyzed by the enzyme nitric oxide synthase (NOS). Endothelial NO synthase (eNOS), is the predominant isoform found in the endothelium, the other two being neuronal NO synthase (nNOS) and inducible NO synthase (iNOS). eNOS requires several co-factors to exert its activity: nicotinamide adenine dinucleotide phosphate (NADPH), flavin adenine dinucleotide (FAD), flavin mononucleotide (FMN), tetrahydrobiopterin (BH4), heme and zinc (Bredt and Snyder 1994). The first step is hydroxylation of L-arginine, and the second step leads to the formation of NO and L-citrulline. Both steps require NADPH, calcium-calmodulin and BH4. Oxygen is required during the second step. Dimerisation of eNOS is required for enzyme activity, and is dependent upon heme, zinc and BH4 (Feletou 2011). Depletion of any of these co-factors can have a negative effect on eNOS activity and NO production.
eNOS activity and therefore the amount of NO synthesised is regulated by a complex integration of transcriptional, post-transcriptional and post-translational pathways. eNOS in endothelial cells is located within the plasma membrane caveolae, an invagination in the plasma membrane rich in cholesterol and spingolipids. Caveolae play a critical role in regulating the activity of eNOS and indeed depleting membrane cholesterol for example reduce eNOS activity by displacing it from the plasma membrane (Qian and Fulton 2013). Post-transcriptional modification of eNOS is also critical to its activity as the enzyme can be palmitoylated, which if prevented by substituting amino acids, results in sub-optimal enzyme activity and decreased NO production. In addition eNOS has multiple phosphorylation sites, one site phosphorylated by for example AKT activation following shear stress or VEGF is important in increasing the activity of eNOS and hence increasing NO production from endothelial cells in response to these stimuli. However, AMPK or PKC-mediated phosphorylation of eNOS at a different site can result in decreased enzyme activity and hence lower production of NO (Qian and Fulton 2013). S-nitrosylation and acetylation of eNOS have also been shown to influence the activity of the enzyme both positively and negatively. Protein-protein interactions can also affect eNOS activity for example the bradykinin receptor acting as an allosteric regulator of eNOS activity. Any one of these pathways may be affected under pathophysiological or toxicological conditions to decrease eNOS activity and increase the risk of hypertension.
Blood vessel relaxation results from NO diffusing from the endothelial cells into the underlying smooth muscle cells, where it activates the enzyme soluble guanylyl cyclase. The latter catalyses the conversion of guanosine triphosphate (GTP) to cyclic guanosine monophosphate (cGMP), which in turn activates cGMP dependent protein kinases. This in turn leads to a reduction of calcium, which restricts contractions, (figure 1.5). NO is a free radical and once produced has a half-life of a few seconds. It reacts with superoxide (Gryglewski, Palmer et al. 1986) and forms peroxynitrite and can also be inactivated by oxyhemoglobin.

Figure 1.5 The production of nitric oxide and resulting vasorelaxation (http://www.downstate.edu/pharmacology/faculty/furchgott.html, accessed 16.03.2015)
Endothelin-1, the most potent vasoconstrictor, is released in response to e.g. angiotensin, and leads to vasoconstriction through activation of G protein-coupled receptors located on smooth muscle cells.

1.6.3 Cardiovascular problems associated with NO deficiency

1.6.3.1 Hypertension

The British Hypertension Society and NICE define hypertension as blood pressure >140/90 mmHg (NICE 2011). The WHO estimated that one billion people lived with uncontrolled hypertension worldwide in 2008. Untreated chronic hypertension increases the risk of cardiovascular diseases including stroke, left ventricular hypertrophy and heart failure, myocardial infarction and sudden death (Aaronson, Ward et al. 2013). There are several recognised risk factors for hypertension: smoking, excessive alcohol intake, physical inactivity, increased BMI and increased sodium intake (NICE 2011).

The pathophysiology of hypertension is multifactorial and includes the involvement of the systems normally regulating the blood pressure: the sympathetic nervous system and the renin-angiotensin-aldosterone system, as well as inflammatory mediators (Schulz, Gori et al. 2011). This leads to inappropriate blood vessel constriction and eventually vascular remodeling, and finally atherosclerosis. On a cellular level, increased oxidative stress leading to endothelial dysfunction is a mechanism often proposed (Apostolova, Blas-Garcia et al. 2011), however it is
debated whether oxidative stress leads to hypertension or vice versa or alternatively both. Certainly increased endothelial cell oxidative stress levels has been linked to decreased NO production by numerous mechanisms including decreasing supply of NO by chemically reacting with it to produce peroxynitrite or decreasing production of NO by inhibiting enzyme activity through affecting phosphorylation status, substrate and co-factor levels, for example.

1.6.3.2 Atherosclerosis

Cardiovascular diseases are diseases of the heart and vasculature and account for one of three deaths, of which half are caused by coronary artery disease (Roger, Go et al. 2012). Coronary artery disease is mostly due to the build up of atherosclerosis in the coronary arteries and takes decades to develop. The arteries are affected a long time before symptoms appear, in fact most remain symptom-free until the lesion produce stenosis > 60 %.

The early stages of atherosclerosis involve the entry, aggregation and binding of small lipoproteins, which are chemically modified, by e.g. oxidative stress in the tunica intima. Oxidative stress leads to the surface of the endothelial cells expressing leukocyte adhesion molecules, which attracts leukocytes. Normal healthy endothelial cells don’t adhere to leukocytes. The leukocyte adhesion molecules fall into two categories, the first being a family of immunoglobulins to which vascular cell adhesion molecule (VCAM-1) belong. VCAM-1 interacts with an integrin, which
is found on T-lymphocytes and monocytes. Selectins is the second class of leukocyte adhesion molecules of which platelet selectin (p-selectin) is believed to play a role in atheroma formation. The leukocytes adhered to the endothelial surface enter the arterial wall in response to chemokines, e.g. monocyte chemoattractant protein-1 (MCP-1), which is produced in response to oxidized lipoproteins. The monocytes are converted to macrophages and take up the oxidized low-density lipoproteins via scavenger receptors, and eventually turn into foam cells. The early atheroma mostly consists of macrophage foam cells, and appears as fatty streaks. The later stages of atheroma formation involves smooth muscle cell migration from the tunica media to the tunica intima in response to chemoattractants from macrophages. The advanced atheroma contains a range of extracellular substances like collagen, proteoglycans and elastin, in addition to lipid laden foam cells.

Atherosclerosis leads to narrowing of the arterial lumen. If stenosis in the coronary arteries restricts blood flow to the myocardium, chronic stable angina results. A consequence following stenosis in other arteries is intermittent claudication on increased demand. Thrombosis due to plaque rupture or plaque erosion in the coronary vessels leads to unstable angina or myocardial infarction.
Risk factors for atherosclerosis include smoking, hypertension, diabetes, obesity, increase cholesterol levels, family history, increasing age, ethnicity and lack of physical exercise.

1.7 Cardiovascular problems in the HIV population

The management of HIV with antiretroviral therapy has led to HIV-infected individuals living a near normal life span. The challenges now facing this population group are associated with age-related illnesses including cardiovascular disease that has been shown to occur more frequently in the HIV population than in the uninfected population (Lepri, Miller et al. 2001). Research into this area has grown as the challenges have been identified with over 6000 articles published about cardiovascular disease and HIV, which reflects the interest and perhaps complexity of the topic.

It is beyond debate that the HIV population is at a higher risk of CVDs than the uninfected population. A recent review of the literature evaluating the risk of cardiovascular diseases (CVDs) showed that the relative risk of CVD was 1.61 (95% CI 1.43-1.81) in HIV infected individuals versus individuals not infected with HIV (Islam, Wu et al. 2012).

Of the cardiovascular diseases, coronary heart disease is of particular concern, which has led to several studies investigating and establishing an increased risk of
MI in the HIV positive population (Grinspoon 2009, Lang, Mary-Krause et al. 2010, Freiberg, Chang et al. 2013). The difference in the prevalence in myocardial infarction between the HIV population and the uninfected population increases with increasing age (Grinspoon 2009) (figure 1.6). Interestingly, HIV infected women versus non infected women have been shown to be at increased relative risk in comparison to HIV infected men versus non infected men (Lang, Mary-Krause et al. 2010).

![Figure 1.6](image)

**Figure 1.6** Myocardial infarction rates in patients diagnosed with HIV (grey line) in comparison to patients not diagnosed with HIV (black line), by age group (x-axis). On the y-axis is the number of events per 1000 person years. Figure from Grinspoon et al (Grinspoon 2009).

Although it has been established that CVDs are more frequent in the HIV population including those on HAART, the reasons behind the increased risk are not clear. The underlying explanation is believed to be multifactorial, with a complex interplay between the chronic inflammation and immune activation subsequent to HIV
infection, overrepresentation of traditional risk factors and exposure to antiretroviral therapy (Fisher, Kanda et al. 2011). The published studies investigating these contributing factors differ in their methodologies, e.g. the large D:A:D study which identified an association between MI and the antiretroviral abacavir did not account for renal function which could have led to selection bias (Strategies for Management of Anti-Retroviral Therapy and Groups 2008). Other studies have not accounted for smoking, e.g. a study that linked the antiretroviral agent efavirenz with myocardial infarction (Durand, Sheehy et al. 2011). CD4 count and viral load, duration of infection and ART use, cocaine and intravenous drug use are other variables with a likely impact on CVD not always adjusted for. The study conclusions should therefore be interpreted with caution. Furthermore the HIV population is still relatively young and therefore the absolute risk of CVDs still remains low. Low cardiovascular event rates, as well as methodological challenges make it difficult to accurately study the impact treatment, the virus itself and the risk factors have upon the development upon CVDs. The three contributory factors are discussed in more depth below.

1.7.1 Traditional risk factors in the HIV population

The HIV population share the same risk factors for CVDs as the general population: smoking, poor diet, high blood cholesterol, diabetes, obesity, insufficient physical activity, alcohol consumption, psychosocial stress and increased blood pressure (NICE 2010). Age as a risk factor is likely to be of increasing significance as the life
expectancy of HIV patients has increased with advances in ART (North and Sinclair 2012). Furthermore, ART and the infection itself may enhance the traditional risk factors.

Smoking has been reported to be 2 to 3 times more prevalent in the HIV population, with up to 60 and 70 % of HIV infected individuals reported as smokers in some studies (Rahmanian, Wewers et al. 2011). In the UK 45 % of HIV infected vs. 35 % in the general population are smokers (Smith, Levy et al. 2004). It is well documented, that cessation of smoking leads to a reduction in coronary heart disease (Critchley and Capewell 2004).

It has also long been recognised that the metabolic syndrome, which refers to the metabolic risk factors; central obesity, high serum triglyceride (TG) levels, low serum high density lipoprotein (HDL) levels, elevated blood pressure and blood glucose levels, is more prevalent in the HIV population group. In fact, 18 % of HIV patients treated with HAART suffer from metabolic syndrome, versus 5 % in the non-infected population (Biron, Bobin-Dubigeon et al. 2012). The lipid profile in this population group is not only altered with regards to increases in TG levels and decreases in HDL, but also decreases in low density lipoprotein (LDL) (Fourie, Van Rooyen et al. 2010). The Swiss HIV Cohort study reported that 26.1 % had elevated
blood pressure in comparison to national target levels (Glass, Ungsedhapand et al. 2006).

The incidence of diabetes in the HIV population versus the uninfected population is debated, with some studies reporting a link between the two (Brown, Cole et al. 2005, Tien, Schneider et al. 2007) and another study reporting no association (Brar, Shuter et al. 2007). A Danish study showed the prevalence of diabetes was increased in HIV infected individuals in the period ranging from 1996 to 1999. Since 1999 however, there has not been an association between diabetes and HIV (Rasmussen, Mathiesen et al. 2012).

In all likelihood, the increased prevalence of some cardiovascular risk factors in the HIV population contribute towards the increased risk of CVD seen in this population group, however even when adjusting for these risk factors there still remains an increased risk of CVDs in the HIV population group.

1.7.2 Contribution of HIV itself to cardiovascular problems

The SMART study (mentioned above) in which patients on episodic ART treatment had a higher prevalence of CVDs than patients on continuous ART suggested that the immunological and inflammatory processes involved in the HIV infection itself contributes to CVDs (Strategies for Management of Antiretroviral Therapy Study, El-
Sadr et al. 2006). Indeed, a follow up study showed a significant increase in the inflammatory marker interleukin 6 and the coagulation marker D-dimer after interruption of ART therapy (Kuller, Tracy et al. 2008). This was related to the increase in viral mRNA. HIV infected participants in The Veterans Aging Cohort study also demonstrated a significantly higher level of interleukin 6 and D-dimer levels than the uninfected veterans (Armah, McGinnis et al. 2012). Both D-dimer and interleukin 6 have been associated with vascular dysfunction in untreated HIV patients (Baker, Quick et al. 2010), and have been shown to be elevated in patients with atherosclerosis (Zakynthinos and Pappa 2009). Indeed, people living with conditions characterized by chronic inflammation e.g. rheumatoid arthritis and systemic erythematous lupus have higher risk of CVD (van Leuven, Franssen et al. 2008).

Elevated levels of biomarkers of inflammation, altered coagulation and monocyte activation e.g. IL-6 and D-dimer, shown to be involved in the development of cardiovascular diseases have also been seen in the HIV population (Armah, McGinnis et al. 2012).

Interestingly, Cui et al. suggested that the secreted HIV protein Nef is involved in the development of atherosclerosis by leading to dyslipidemia, and accumulation of
cholesterol in macrophages resulting in the formation of foam cells in the vessel wall (Cui, Ditiakovski et al. 2014).

1.7.3 Contribution of ART to cardiovascular problems

The Data Collection on Adverse events of Anti-HIV Drugs (D:A:D) is a prospective, multi cohort study following one of the largest databases of HIV infected individuals, which has resulted in several landmark studies that have shaped clinical prescribing practice. Their first publication which linked the use of ART with myocardial infarction (Friis-Moller, Sabin et al. 2003) was followed by a publication in which not only myocardial infarction, but also other cardio and cerebrovascular diseases were associated with ART use (d'Arminio, Sabin et al. 2004). Later studies identified specific HIV drug classes with myocardial infarction: PIs such as lopinavir with ritonavir (Group, Friis-Moller et al. 2007) and NRTIs: specifically didanosine and abacavir (Group, Sabin et al. 2008). Nevirapine and efavirenz, the two NNRTIs included in the D:A:D studies were not linked with an increase risk of myocardial infarction (Group, Friis-Moller et al. 2007, Worm, Sabin et al. 2010), however a later Canadian study did find an association between efavirenz and myocardial infarction (Durand, Sheehy et al. 2011). Several other studies have confirmed a link between ART and increased risk of CVDs, e.g. a review by Islam et al., showed an increase in the relative risk of 1.52 (95 % CI, 1.35-1.70) in HIV patients on ART versus HIV infected patients that were treatment naïve. The highest risk was associated with PIs followed by NRTIs and NNRTI (Islam, Wu et al. 2012). It is noteworthy that HIV
treatment is guided by CD4 count and only initiated once the immune system is weakened. Treatment experienced patients have been infected with HIV for a longer time period, in comparison to treatment naïve patients. This may partially explain why HIV patients receiving treatment have a higher risk of CVD than treatment naïve patients. The SMART study, in which all participants were on ART, showed that intermittent use of ART was associated with a higher degree of CVDs than continuous use (Strategies for Management of Antiretroviral Therapy Study, El-Sadr et al. 2006). This study indicates that ART has less detrimental effects on the CVS than the virus itself. Other studies, e.g. the HOPS study did not find a statistically significant contribution of ART to cardiovascular diseases (Lichtenstein 2006).

It is important to recognise that although ART has been shown to increase the risk of CVDs in some studies, the absolute risk of e.g. myocardial infarction is still low and the benefits of ART outweighs the risks.

The link between CVDs the NNRTIs efavirenz, rilpivirine and etravirine, and the NRTI abacavir is further discussed in the relevant results chapters.
1.8 Possible mechanisms behind damaging cardiovascular effects of ART

Oxidative stress, endoplasmic reticulum stress, endothelial dysfunction. PARP activation and triggering of apoptotic and necrotic cell death pathways have been associated with antiretroviral damage on a cellular level.

1.8.1 Oxidative stress

Cells produce highly reactive reactive oxygen species (ROS) as a part of fulfilling normal physiological functions. There is a homeostatic balance between ROS and antioxidants, maintaining the levels of ROS relatively constant. At lower ROS concentrations, beneficial effects are seen e.g. in defending the body against infectious organisms. If the ROS concentration is too high due to higher ROS production and/ or disturbances to the ROS/antioxidant balance, the ROS can lead to cellular damage, at which point it is termed oxidative stress.

The superoxide anion (O$_2^-$) is the first ROS to be produced which results from metabolic activities or irradiation. The mitochondria are the main site of production, due to electrons escaping the mitochondrial electron transport chain and reacting with oxygen thereby forming superoxide. Other cellular sources of superoxide anion include xanthine oxidase, NAD(P)H oxidase, protein kinase C, etc.
Superoxide dismutases (SOD) are enzymes, which catalyse the reaction (dismutates) of the superoxide anion to oxygen and hydrogen peroxide (H\textsubscript{2}O\textsubscript{2}) a reactive oxygen species that is not a free radical. The Fenton reaction, in which a metal like Fe\textsuperscript{2+} is oxidised to Fe\textsuperscript{3+} leads to breakdown of hydrogen peroxide to the hydroxyl radical (HO\textsuperscript{-}). Fe\textsuperscript{3+} is reduced to Fe\textsuperscript{2+} by superoxide. The hydroxyl radical is particularly reactive and reactions therefore occur near the site of the free radical production.

The hydroxyl radical is converted to a lipid radical by extraction of an electron from polyunsaturated lipids. The lipid radical is further converted to a lipid peroxyl radical by reacting with oxygen. If the lipid peroxyl radical is not reduced by antioxidants it goes down one of two lipid peroxidation pathways. The lipid peroxyl radical can undergo two cyclisations: coupling to oxygen and a reduction reaction to yield malondialdehyde, MDA, which is mutagenic in human cells. The second lipid peroxyl pathway leads to the formation of 4-hydroxynonenal, which is toxic to vascular cells.

Members of the three main groups of ART; NRTIs, NNRTIs (including efavirenz) and PIs have all been associated with an increase in ROS. NRTI was the first ART group to be associated with oxidative stress in 2004 (Day and Lewis 2004), due to these drugs not only being competitive inhibitors of HIV reverse transcriptase but also
they were found to inhibit DNA polymerase-γ in the mitochondria. Inhibition of DNA polymerase-γ results in mitochondrial toxicity and increased cellular oxidative stress. More recently first generation NNRTIs such as efavirenz have been shown to increase oxidative stress in two cell types: human coronary artery endothelial cells (Jamaluddin, Lin et al. 2010), and hepatocytes (Apostolova, Gomez-Sucerquia et al. 2010), this has also been linked to mitochondrial toxicity but NOT through inhibition of DNA polymerase-γ but rather by direct inhibition of mitochondrial complex I. The effects of the NNRTIs rilpivirine and etravirine on the production of ROS in endothelial cells have not yet been established, nor has the effects of the NNRTIs on ROS production been determined in cardiac cells.

Oxidative stress in turn has been linked to endothelial dysfunction, which in turn has been linked to hypertension and atherosclerosis (Siti, Kamisah et al. 2015).

**1.8.2 Endothelial dysfunction**

The endothelium together with the underlying smooth muscle regulates vascular tone. Endothelial dysfunction results due to an imbalance between vasodilating and vasoconstricting compounds. Endothelial dysfunction therefore leads to an impairment of the ability of the arterial side of the circulation to dilate fully and/or an increased response to vasoconstricting compounds (Davel, Wenceslau et al. 2011).
A reduction of NO bioavailability is a common characteristic of endothelial dysfunction and can be due to decreased production of nitric oxide, an imbalance between reactive oxygen species (ROS) production and antioxidant defence mechanisms and therefore inactivation of NO by ROS, a decrease in eNOS gene and protein expression or a decrease in the concentration of the building blocks required to make NO. The resulting decrease in NO leads to an inability of the vasculature to dilate fully. The endothelium also becomes more pro thrombotic.

PIs, including abacavir, have been shown to cause endothelial dysfunction in porcine coronary arteries. A reduction in relaxation and contraction was seen, eNOS mRNA levels were reduced and superoxide levels increased (Chai, Yang et al. 2005). The NNRTI efavirenz has been shown to lead to endothelial dysfunction in our lab (Faltz 2013).

Traditional risk factors for cardiovascular diseases e.g. hypertension, atherosclerosis and diabetes are associated with endothelial dysfunction (Davel, Wenceslau et al. 2011).

**1.8.3 Poly (ADP-ribose) polymerase-1 (PARP) activation**

PARP is group of enzymes with a range of different roles within the cell, including DNA damage detection and repair. PARP-1 is found in the nucleus and contains several domains: DNA binding domain, a catalytic domain and an auto modification
domain. A caspase-cleavage site lies within the DNA binding domain (Krishnakumar and Kraus 2010). PARP-1 is activated in response to damage to DNA, and the DNA binding domain binds to the damaged areas of the DNA. The catalytic part is responsible for synthesis of a poly ADP-ribose chain (PAR), which acts as a signal attracting other molecules involved in the DNA repair. When there is extensive damage to DNA, a large amount of ADP-ribose monomers are synthesized. NAD+ is required as a substrate in the synthesis of ADP-ribose. Extensive DNA damage therefore leads to extensive poly (ADP-ribose) synthesis and NAD+ depletion. As the cells require NAD+/NADPH to generate ATP via oxidative phosphorylation, depletion of ATP results. This in turn leads to necrotic cell death. Caspase 3 and 7 are able to cleave PARP-1 creating two fragments inactivating PARP and leading to cell death through the apoptotic pathway (Los, Mozoluk et al. 2002) (figure 1.7).

Figure 1.7 PARP involvement in apoptosis and necrosis (http://uts.cc.utexas.edu/~liulab/research.php?id=4, accessed 24.04.2015)
Treatment with efavirenz has been shown to cause overactivation of PARP (Faltz 2013), an effect that has also been seen with the NRTI zidovudine (Szabados, Fischer et al. 1999).

1.8.4 Apoptosis and necrosis

Apoptosis and necrosis are two distinctively different forms of cell death. Apoptosis is a normal physiological response, which is involved in the development and homeostasis of tissues and is advantageous to the cell. It is commonly referred to as programmed cell death and leads to characteristic cell changes that include chromatin condensation, cell membrane blebbing and the formation of apoptotic bodies (Newmeyer and Ferguson-Miller 2003). Apoptosis can occur through several different pathways and is a multistep process. It involves the mitochondria, and can either be extrinsic in which case it is initiated by death receptors on the plasma membrane or it can be intrinsic which is caused by intracellular homeostatic disturbances and involves the BCL-family of proteins. The intrinsic pathway leads to an increase in mitochondrial permeability, and consequently the movement of cytochrome C, small mitochondria-derived activators of caspases (SMAC) and/or apoptosis-inducing factor (AIF) from the mitochondrial department to the cytosol. This in turn leads to apoptosis either via caspase cysteine proteases or through a caspase-independent pathway. The extrinsic pathway activates caspase-8 directly with mitochondrial amplification further downstream. The apoptotic process is
rigorously controlled, however can be inappropriately induced and lead to pathologies.

Cell death by necrosis on the other hand, results from injury to the cell by e.g. toxins, stress or pathogens, and is viewed as deleterious. Traditionally necrosis was described as uncontrolled cell death, however in the last few years it has become apparent that necrosis is a tightly regulated mechanism (Golstein and Kroemer 2007). It differs from apoptosis in that it doesn't appear to have a role in normal physiological development, and involves the opening of mitochondrial permeability transition pores (MPTP) which in turn disturbs mitochondrial respiration and leads to cell death due to ATP deficiency (Dorn 2013).

Several HIV drugs have been shown to lead to apoptosis in different cell types *in vitro*, including treatment with efavirenz (Pilon, Lum et al. 2002, Faltz 2013).

Apoptosis has been shown to be involved in cardiovascular diseases, e.g. myocardial infarction, reperfusion injury and cardiac failure (Kim and Kang 2010).

1.8.5 *Endoplasmic reticulum stress*

The endoplasmic reticulum (ER) is a multifunctional organelle found in eukaryotic cells where its functions include synthesis, folding, modification and transport of secretory and membrane proteins as well as calcium homeostasis and lipid
synthesis. The functions of the ER have recently been reviewed in detail (Lynes and Simmen 2011). Nearly all the proteins that are synthesized are translocated to the ER for further modification. In the heart, the ER is known as sarcoplasmic reticulum (SR) and it specializes in the regulation of calcium. The ER communicates with the nucleus, plasma membrane and the mitochondria.

A range of different stresses from different sources can lead to perturbation of the ER environment. Examples are oxidative stress, radiation, hypoxia, heat, glucose deprivation or dysfunction of the protein glycosylation machinery, disturbance to the calcium regulation, viral infection and impairment of protease function. An oxidising environment is important for the formation of disulfide bonds within the protein, which is necessary for the proper folding of the protein, i.e. the amino acid chain acquiring their unique three-dimensional structure. The correct calcium level is important as resident proteins (ER chaperones) that assist in the proper folding of the newly synthesised proteins rely on calcium for their functioning. Several ER chaperones have been identified, e.g. binding protein/glucose regulated protein BiP/GRP78, calnexin and calreticulin. The folding of proteins is particularly susceptible to disturbances to the ER environment and the latter lead to an accumulation of misfolded proteins, proteins that do not have the appropriate three-dimensional shape. This is known as ER stress. Several pharmacological agents are used experimentally to induce ER stress. Tunicamycin induces ER stress
via reduction of protein glycosylation, thapsigargin by reducing the concentration of calcium in the ER and dithiothreitol alters the redox status within the ER.

ER stress rapidly activates a complex signalling pathway known as the unfolded protein response (UPR), which attempts to deal with increased levels of incorrectly folded proteins and restore a homeostatic ER environment. This was first described in 1988 by Kozutsumi et al who noticed an increase in glucose-regulated proteins in response to misfolded proteins in the ER (Kozutsumi, Segal et al. 1988). During the pro-survival phase of ER stress the net result is communication between the ER and nucleus, which lead to an upregulation of genes encoding ER chaperones and downregulation of genes encoding a range of other proteins thereby transiently increasing the protein folding capacity and reducing the workload of the ER respectively. Furthermore non-functional proteins that are incorrectly folded are transported from the lumen of the ER to the cytosol where they are “tagged” for proteasomal degradation by ubiquitin proteins attaching to them. This is known as ER-associated degradation (ERAD).

Prolonged or overwhelming ER stress in which a homeostatic ER environment is not re-established results in programmed cell death. This stage of ER stress is often referred to as the pro-apoptotic phase (figure 1.8).
Figure 1.8 Figure shows the different stages of ER stress. Initially the signalling pathways activated by ER stress aim to restore a homeostatic ER environment. If ER stress is excessive or prolonged and remain unresolved, apoptotic cell death pathways are activated (Glembotski 2007).

Initially three transmembrane proteins are activated during ER stress. Protein-kinase-like RNA ER kinase (PERK) and activating transcription factor 6 (ATF6) are activated by their dissociation from glucose regulated protein 78dKA (GRP78), which under homeostatic ER conditions maintain these proteins in their inactive
state by binding to them. The third transmembrane protein, inositol-requiring kinase 1 (IRE1α) is activated directly by an increase in unfolded proteins which leads to oligomerization of IRE1α and autophosphorylation.

PERK exists as a monomer under unstressed conditions, and is attached to GRP78 on the luminal side (Bertolotti, Zhang et al. 2000). ER stress leads to dissociation of PERK from GRP78 and dimerization. This is closely followed by autophosphorylation and activation. GRP78 relocates to the unfolded proteins in the ER where it acts as an ER chaperone. Activated PERK leads to phosphorylation of ribosomal elongation factor 2α (eIF2α) and increased transcription of factor ATF4. The former decreases cap/ eIF2α dependent mRNA translation, which reduces the protein folding workload of the ER by inhibiting protein synthesis. Transient induction of ATF4 induces genes encoding for ER chaperones and amino acid transporters that are involved in resolving the ER stress. These proteins escape PERK mediated translational inhibition by possessing specific structural feature. Transient ER stress leads to transient protein synthesis reduction. Translation resumes when cellular stresses diminish. This occurs during the prosurvival phase of ER stress (figure 1.9)

The second transmembrane protein, ATF6 exists as a dimer bound to GRP78 during homeostatic ER conditions. ER stress leads to separation of GRP78 from the ATF6 dimer by active dissociation (Shen, Snapp et al. 2005) and cleavage of disulfide bond
linking the two ATF6 molecules thereby relieving the ATF6 monomer, p90, which is translocated from the ER to the lumen of the Golgi complex. Proteolytic cleavage of the monomer reveals an active fragment of ATF6, N-ATF6 or p50. N-ATF6 translocates to the nucleus and activates a range of genes involved in the UPR eg. GRP78, GRP94, calnexin and CHOP (figure 1.9).

Direct interaction with unfolded proteins leads to dimerization, autophosphorylation and activation of the third transmembrane protein: IRE1α (Shamu and Walter 1996). Autophosphorylation activates the endoribonuclease activity (Sidrauski and Walter 1997), which in turn leads to splicing of the mRNA that encodes the transcriptional factor X-box binding protein 1 (XBP1). Activated XBP1 regulates several UPR-related genes that are involved in restoring ER homeostasis. IRE1α also activates Apoptotic-Signalling Kinase-1 (ASK1), which in turn activates J Jun-N-terminal kinase (JNK) and p38 MAPK, which leads to activation of CHOP (figure 1.9).
Figure 1.9  The signalling pathway of ER stress following activation of PERK, IRE1α and ATF6 (Glembotski 2007).
Prolonged ER stress leads to programmed cell death, of which the leucine zipper transcriptional factor C/EBP homologous protein (CHOP) also known as GADD153 is up-regulated and leads to an increase in several pro apoptotic proteins and a decrease in the level of anti-apoptotic proteins.

Cardiovascular pathologies associated with ER stress include cardiac hypertrophy and heart failure (Hamada, Suzuki et al. 2004, Okada, Minamino et al. 2004), the development of artherosclerosis and cardiac damage after ischaemia/reperfusion.

Treatment with efavirenz in vitro has shown to cause ER stress in both hepatocytes (Apostolova, Gomez-Sucerquia et al. 2013) and H9c2 cells (Faltz 2013). Several PIs such as ritonavir and lopinavir have been linked to ER stress in different cell types including human endothelial cells (Zhou, Pandak et al. 2005, Zhou, Gurley et al. 2006, Sun, Wu et al. 2009, Wu, Sun et al. 2010, Zha, Wan et al. 2013).

1.9 Aims of study
The overall objective of this study was to further research the effect of antiretroviral drugs on cells of the cardiovascular system. HIV patients take these medications for extended periods of their lives; evidence of possible cardiovascular side effects via ART-mediated cellular dysfunction may be clinically relevant.
To this end, the first aim of this study was to evaluate the cardiovascular cellular effects of the second generation NNRTIs: etravirine and rilpivirine, and compare them to those of efavirenz *in vitro* and *ex vivo*. A large amount of work has already been published evaluating the effects of the first generation NNRTI efavirenz on endothelial and cardiac cell function however no research has yet been undertaken to examine the effects of second generation NNRTIs. Using an endothelial and myocardial cell line and *ex-vivo* rat aortic rings we will determine whether second generation NNRTIs have a better toxicity profile *in vitro* than the first generation by assessing cell viability and mode of cell death (apoptosis and/or necrosis), cellular oxidative stress, levels of ER stress, inflammation via IL-8 release and cell function via NO production. Underlying cellular mechanisms of any dysfunction mediated by second generation NNRTIs will be elucidated using appropriate pharmacological tools.

The second aim was to evaluate the *in vitro* cardiotoxic effects of the NRTI abacavir. Abacavir has already been shown to increase the risk of myocardial infarction in HIV patients although the cellular mechanism underlying this pathology is still unknown. Abacavir has been linked to loss of endothelial cell function *in vitro*, however, direct effects of abacavir on cardiac myocytes *in vitro* have yet to be investigated. Using a myocardial cell line we will therefore assess the effects of abacavir on cell viability, apoptosis and necrosis levels as well as ER stress. Abacavir may not have a direct toxic effect on cardiac myocytes but increase their
susceptibility to known cellular damaging agents linked to increased risk of myocardial infarction such as hypoxia and lipotoxicity. We will also examine the effects of abacavir on the toxic effects of hypoxia induced chemically by cobalt chloride and lipotoxicity induced by palmitic acid to determine whether abacavir is able to enhance their cellular damaging profile.
Chapter Two: **Material and methods**
2.1 Materials

**Abcam (Cambridge UK):** NFκB p65 SimpleStep ELISA Kit

**BD Bioscience (Oxford, UK):** Human IL-8 ELISA kit, TMB Substrate Reagent Set

**BioRad (Hertfordshire, UK):** Laemmli buffer, 2 mercaptothanol, Precision Plus Protein Standards, filter paper

**Calbiochem (Nottingham, UK):** PARP inhibitor PJ-34, PERK inhibitor GSK 2606414

**Cayman chemicals (via Bioscience, Oxford, UK):** Abacavir

**European Collection of Cell Cultures (ECACC, Salisbury, UK):** H9c2 cells, EA.hy926 cells

**GE Healthcare:** Non-essential amino acids, ECL prime Blotting Detection System,

**PAA (Slough, UK):** DMEM, trypsin

**Promega (Southampton, UK):** Caspase Glo 3/7 kit

**Roche (Welwyn Garden City, UK):** Bovine serum albumin, Cell death kit

**Sainsburys (Brighton, UK):** Dried skimmed milk powder

**Santa Cruz Insight Biotechnology (Wembley, UK):** β-Actin primary antibody monoclonal IgG1, GADD 153 primary antibody mouse monoclonal IgG1, primary antibody, goat anti-mouse antibodies.

**Sequoia Research (Pangbourne, UK):** Efavirenz, etravirine, rilpivirine

**Sigma Aldrich (Poole, UK):** Acetylcholine, cobalt chloride, ammonium persulfate, TEMED, dimethylsulfoxide, Tween 20, palmitic acid, 2.7-dichlorofluorescein (DCF), propidium iodide, nitrotetrazolium blue (NBT), acrylamide/bisacrylamide 30 %
solution, MTT, TRIZMA base, sodium deoxycholate, Hoescht 33342, Coomassie Brilliant Blue

Whatman (Maidstone, UK): Protran Nitrocellulose transfer/blotting membrane

General lab supplies obtained from Fisher Scientific (Loughborough, UK)

2.2 Buffers and solutions

Krebs Buffer

150 mM NaCl
1.17 mM MgSO$_4$
14.9 mM NaHCO$_3$
4.7 mM KCl
1.18 mM KH$_2$PO$_4$
5 mM Glucose
1.6 mM CaCl$_2$

Phosphate buffered saline (PBS)

137 mM sodium chloride
2.7 mM potassium chloride
10 mM disodium hydrogen phosphate
2 mM potassium dihydrogen phosphate
Radio-immuno precipitation assay (RIPA) buffer

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

Running Buffer x 10

250 mM Tris
1920 nM glycine
1 % SDS

Separating gel (10 % gel)

3.3 ml acrylamide (30 %/Bis
4.3 ml deionized water
2.5 ml 1.5 M TRIS ph = 8.8
100 µL ammonium persulfate
14 µL TEMED
Stacking gel

830 μL acrylamide (30 %)/Bis
2.8 ml deionied water
1.26 ml 0.5 M TRIS pH = 6.8
50 μL 10 % ammonium persulfate
50 μL 10 % SDS
10 μL TEMED

TBS Tween

10 % TBS x 10 stock solution
90 % deionised water
0.05 % Tween 20

Transfer buffer

10 % 10x running buffer
20 % methanol
70 % distilled water

Tris buffered saline (TBS) x 10

500 mM Tris
1500 mM NaCl
Adjust pH to 7.6 with HCl
2.3 Methods

2.3.1 Cell culture

H9c2 cells and EA.hy926 were obtained from the European Collection of Cell Cultures in frozen vials and thawed out following the recommended procedure. The vials were placed in a 37 °C water bath until thawed (approximately 2 minutes). The content was moved to a centrifugation tube with 9 ml media and centrifuged at 125 g for 5 minutes. The media was removed and the pellet re-suspended in culture media (see below) and split into a single use 75 cm² polystyrene flask (Corning).

The H9c2 cell line is derived from embryonic rat cardiac tissue and has maintained many of the characteristics of myocardial cells, and is therefore frequently used to study the direct effects drugs have on cardiac myocytes (Enayetallah, Puppala et al. 2013).

The EAhy926 cell line is one of very few the human macro vascular cell lines and is a fusion between human umbilical cord endothelial cell and human lung carcinoma cell line A549 (Bouis, Hospers et al. 2001). However, the EA.hy926 cell line shares many characteristics of primary endothelial cells including Weibel-Palade bodies in the cytoplasm, in which both interleukin 8 and endothelin are stored, the endothelin converting enzymes, and tissue specific organelles (Ahn, Pan et al. 1995). In addition the EA.hy926 cell line displays characteristics of endothelial cell functions including angiogenesis, homeostasis/thrombosis and inflammation (Edgell, McDonald et al.
However, they do have one major limitation in that their production of nitric oxide is very low though they do express the eNOS enzyme (Su, Qadri et al. 2013).

The work presented in this thesis carries on from work previously done in our laboratory on the H9c2 and EA.hy926 cell lines. Passage 6 to 50 were used to study the behaviour of cardiac myocytes and endothelial cells in response to different treatments.

The cell lines were grown as an adherent monolayer in 75 cm² single-use sterile polystyrene flasks in a humidified incubator (Heraeus) where the physiochemical environment was kept constant (95 % air, 5 % CO₂, 37 °C). The cells were grown in Dulbecco's Modified Eagle's Medium (DMEM) with 4 mM L-glutamine, 4.5 g/L glucose, 1 mM sodium pyruvate, which was supplemented with heat inactivated 10 % fetal bovine serum 1 % non-essential amino acids, 100 U/ml penicillin and 50 µg/ml streptomycin (culture media).

When the cells reached approximately 80 % confluence (approximately every third or fourth day) they were passaged aseptically in a class II laminar flow cabinet. The monolayer of cells was incubated with a 0.1 % w/v trypsin-EDTA (0.02 % w/v) for approximately 5 minutes, in order for the cells to detach from the polystyrene surface. The trypsin was then neutralized with an equal volume of culture media.
and the cell suspension centrifuged for 5 minutes at 500 g. The supernatant was removed and the cell pellet re-suspended in culture media. The cell suspension was then divided between 3 (H9c2) or 4 (EA.hy926) single use 75 cm² polystyrene flasks and another 12 ml of media added to each flask. The culture media was replaced every 2 days, between passaging.

Plating for cell death, MTT, NBT, DCF and ELISA assays in 6, 12, 24 or 96 well micro-titration plates were also done aseptically in a class II laminar flow cabinet. The cell number was determined using a haemocytometer and the media differed to the culture media in that it was supplemented with 3 % FBS rather than 10 % (treatment media).

### 2.3.2 In vitro treatments

Stock solutions of efavirenz, etravirine and rilpivirine were prepared by dissolving the drug in DMSO and storing at room temperature. The different concentrations employed in the different experiments were prepared by diluting the appropriate stock solution in treatment media. The DMSO concentration was less than 0.3 % in the different experiments, and had no effect on the parameters measured. Abacavir was obtained from Cayman Chemical Company. Stock solutions were prepared by dissolving abacavir powder in distilled water, which was diluted in treatment media to the required concentrations.
Steady state $C_{\text{max}}$ for etravirine following administration of 200 mg twice daily was reported to be 1001 ng/mL (2.3 mM) (Boffito, Jackson et al. 2009), 4.0 $\mu$g/mL (12.6 mM) for efavirenz following 600 mg daily, and 296 ng/ml (0.81 mM) for rilpivirine following administration of 75mg (Crauwels, van Heeswijk et al. 2008). 6.9 $\mu$M has been reported as the $C_{\text{max}}$ for abacavir (DiCenzo, Forrest et al. 2003).

High HIV mutation rates result in the continuous development of new HIV strains, of which there are many. A drawback of the quantitative structure activity relationship models which determine IC$_{50}$ values is that they predict the biological activity of drugs against only one species of the virus, and often only for a few HIV drugs which make comparisons difficult. An examples of IC$_{50}$ for wild type HIV reverse transcriptase in which all three NNRTIs have been tested: efavirenz 2 nm, etravirine 1 nm and rilpivirine 1 nm (http://pubchem.ncbi.nlm.nih.gov/assay/assay.cgi?aid=1069125, accessed: 26.04.2015). IC$_{50}$ values for efavirenz for different strains have been reported to be between 0.9 and 3 nM (Young, Britcher et al. 1995, Kollmann, Tremblay et al. 2001), however in the presence of specific mutations the IC$_{50}$ values vary greatly, e.g IC$_{50}$ value for efavirenz in the presence of the Y181C mutation in reverse transcriptase is 126 nM (http://pubchem.ncbi.nlm.nih.gov/assay/assay.cgi?aid=573466#aDescription, accessed: 26.04.2015). The IC$_{50}$ value has been reported to be 130 nM against a wild type HIV reverse transcriptase
To compare the different NNRTIs, the concentrations used in the work presented in this thesis were based on three factors. Firstly trying to replicate long-term exposure to the drugs by using higher concentrations than the $C_{\text{max}}$ for a short period (up to 48 hours), secondly it was based on previous work carried out on efavirenz in our lab (Faltz 2013) and thirdly the concentrations have been used in other publications (Apostolova, Gomez-Sucerquia et al. 2013). Abacavir concentrations were based on the abacavir $C_{\text{max}}$ and concentrations used in work previously published (Wang, Chai et al. 2009, De Pablo, Orden et al. 2010).

2.3.3 Cell viability assay

The MTT assay was chosen to assess cell viability as it reflects not only cell viability but can also be used as a crude measurement of mitochondrial activity, as the reduction of MTT to a purple formazan product is mainly a result of mitochondrial dehydrogenases of metabolically active cells (Brand and Nicholls 2011). The MTT assay is a colorimetric assay in which water soluble yellow 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) is reduced to the water insoluble purple MTT formazan by reductase enzymes of viable cells. An organic solvent is used to dissolve the dye and a spectrophotometer quantifies the absorbance of the solution, which is an estimate of cell viability (van Meerloo, Kaspers et al. 2011).
Monolayers of EA.hy926 and H9c2 cells in 96-well microtitration plates were incubated (37 °C, 5% CO₂) and allowed to grow for 24 hours in media containing 3% FBS before exposing the cells to different concentrations of different treatments in 3% FBS containing media. 100 μL of solution was added to each well. The drug solution was removed after a 24 hour incubation period and replaced with a 1 mg/mL solution of MTT in treatment media. The microtitration plates were incubated for a further 30 minutes. The MTT solution was then removed and 100 μL dimethyl sulfoxide (DMSO) was added to the wells in order to solubilize the MTT formazan. The absorbance of the purple MTT solution was quantified by an Oasys UVM 340 spectrophotometer at 540 nm. The measurements of the treated cells are expressed as a percentage of the untreated cells (control) and indicates loss of cell viability. The method was adapted from van Meerloo, Kaspers et al. (van Meerloo, Kaspers et al. 2011).

2.3.4 Evaluation of apoptosis and necrosis using propidium iodide/ Hoescht (HPI) staining

Morphological analysis of classic apoptosis and necrosis characteristics was chosen as a method to determine the levels and cellular mechanism of cell death as it is sensitive, rapid and convenient. The simultaneous use of the dyes propidium iodide and Hoescht 33342 results in a staining pattern allowing normal, apoptotic and dead cell populations to be distinguished via fluorescence microscopy (Harrison,
Propidium iodide is a fluorescent dye, which does not permeate the cell membrane of living cells, and it is therefore used to identify cells with a damaged cell membrane (necrotic cell membrane). Once inside the cells, the dye binds to the DNA by intercalating between the DNA bases. This leads to a 20- to 30-fold increase in the fluorescence. Cells stained with propidium iodide fluoresce red. Hoescht 33342/33258 (461 nm) is a fluorescent dye, which binds to the DNA and emit a blue fluorescent light. A brief review of different methods to detect apoptosis have been published by Ulukaya et al. (Ulukaya, Acilan et al. 2011).

EA.hy926 and H9c2 cells were plated in 24 well plates, each well containing approximately 4 x 10^4 cells. Following a 24 hour incubation period, the cells were exposed to different treatments in media containing 3 % FBS, and incubated for a further 24 hours. The cells were then stained with 50 μL Hoescht (1 μg/mL) and propidium iodide (2 μg/ml) dissolved in media and incubated for a further 10 minutes.

The cells were counted using an Axiovert 25 microscope under 320x magnification, excitation wavelength 365 nm and emission wavelength 420 nm. Living cells emitted a blue fluorescent light whereas necrotic cells emitted a red fluorescent light. Apoptotic cells were stained either blue or red, and have a distinct appearance with the cells having at least 3 apoptotic bodies.
Approximately 300 cells x 2 were counted for each concentration/control per experiment. Each experiment was repeated three times and living cells, necrotic cells and apoptotic cells were expressed as a percentage of the untreated cells.

2.3.5 Evaluation of apoptotic cell death

In addition to morphological staining of cells to determine levels of apoptosis a second method was used to confirm the observations, to that end a commercially available ELISA that measures the levels of histones in the cytoplasm was utilised. Histones are usually only found in the nucleus in normal cells and only under very few conditions are they found in the cytoplasm, apoptosis being one of those conditions. The Cell Death Detection Elisa that measures cytoplasmic histones and hence give an assessment of apoptosis was obtained from Roche Diagnostics. The protocol supplied with the kit was followed. Briefly, H9c2 cells were plated in 96 well plates and incubated with various treatments for 24 hours. The supernatant was removed and the cells lysed by adding the supplied lysis buffer for 30 minutes. The lysate was then centrifuged at 200 x g for 10 minutes. 20 μl of the supernatant (cytoplasmic fraction) was removed and added to the supplied ELISA plate. 80 μl immunoreagent was added to each well. The plate was covered with foil and placed on a shaker (50 rpm). After a 2 hour incubation period the supernatant was removed and each well rinsed with 250 μl incubation buffer three times. ABTS solution was added and the plate left until a colour change was seen (20 minutes).
100 μl stop solution was added and the wavelength measured at 405 nm. Data is expressed as percentage of untreated cells.

2.3.6 Determination of activation of caspase-3/7

Cellular apoptosis can be mediated through caspase dependent or independent mechanisms. Caspase-3/7 activation is observed in caspase-dependent apoptosis being key regulators of the process hence measurement of these two enzymes was chosen as a method to determine if any observed apoptosis was being mediated through the caspase dependent pathway (Hongmei 2012). Caspase-3/7 activity was measured with a luminescent assay obtained from Promega. The protocol supplied with the kit for cells cultured in 96 well plates was followed. Briefly, EA.hy926 and H9c2 cells were plated in 96 white walled well plates (10 000 cells/well), and exposed to various treatments for 24 hours. The supplied Caspase-Glo Substrate was mixed with Caspase-Glo Buffer, and the resulting reagent mixture was added to each well (100 μl). The luminescence was measured after 1 hour incubation (37 °C, 5 % CO2) with a plate reading luminometer. Data is expressed as percentage of untreated cells.

2.3.7 Measurements of interleukin 8

Measurement of Interleukin 8 (IL-8) release from endothelial cells was used as a marker of inflammation as it has been shown to be a key player in the development of atherosclerosis. IL-8 has been identified at sites of vascular injury where its role
has been shown to be chemotactic enhancing the adherence of rolling macrophages to endothelial cells expressing E-selectin as well as playing a direct atherogenic role stimulating vascular smooth muscle proliferation. IL-8 secreted by EA.hy926 cells was measured with an IL-8 kit from BD Biosciences. The manufacturers recommended assay procedure was followed. Briefly, the cells were plated in 48 well plates and incubated for 3 days, treated with different drug solutions and incubated for a further 24 hours. 96 well ELISA plates were coated with Capture Antibody diluted in Coating Buffer (100 μl per well), sealed and incubated overnight. The wells were aspirated and washed 3 times with 300 μl wash buffer. 200 μl assay diluent was added to each well and the plate incubated at room temperature for a further 1 hour. The wash step was repeated, and 100 μl supernatant from the drug treated plates added to each well in the ELISA plate. After a 2 hour incubation period, the ELISA plate was washed (five washes) and Working Detector added to each well. The ELISA plate was covered and incubated for one hour at room temperature. The wash step was repeated (7 washes) and 100 μL Substrate Solution was added to each well. After a 30 minute incubation period in the dark, 50 μl Stop Solution was added to each well and the plate read at 450nm. The samples were diluted with assay diluent as necessary. A standard curve for IL-8 was prepared and the amount of protein in the sample plates determined by the Bradford assay, described below. Data is expressed as amount of interleukin 8 (pg) in relation to amount of protein (mg).
2.3.8 Measurements of endothelin

Increased release of endothelin has been linked to hypertension and cardiovascular disease (Kolettis, Barton et al. 2013), hence this is a potential cellular pathway affected by antiretroviral drugs. Therefore, release of endothelin from endothelial cells was measured using an ELISA kit supplied by Caymen Chemical (kit 513151). Briefly, EA.hy926 cells were treated with various treatments for 24 hours. 100 μl of treatment media was added to each well in the ELISA plate pre-coated with monoclonal antibody specific for endothelin. Each sample was analysed in duplicate. 100 μL AChE Fab conjugate (which binds to endothelin) was added to each well and the plate incubated overnight at 4 ºC. The wells were washed with Wash Buffer five times after the incubation period, and 200 μl Ellman’s reagent added to each well. Ellman’s reagent contains a substrate for AChF, and the concentration of endothelin is determined by measuring the enzymatic activity of AChE. The plate was left for 2 hours in the dark and absorbance read at 420 nM following the incubation period. The results are expressed as percentage of untreated cells.

2.3.9 Detection of NFκB

The transcription factor NFκB is a key mediator of inflammation in many cell types including those of the vasculature and indeed has been shown to mediate the increased IL-8 release from endothelial cells (Fiuza, Bustin et al. 2003). Therefore, this transcription factor was chosen as a marker to determine whether antiretroviral drugs have transcriptional effects in endothelial cells. NFκB (p65)
activation was measured with an ELISA assay for p65 obtained from abcam and the supplied protocol was followed. Briefly, EA.hy926 cells were treated with various treatments for 1, 2 and 4 hours, the treatment media removed and the adherent cells solubilised and lysed. The lysate was added to the ELISA plate, alongside positive controls, and incubated overnight. Following a wash step, NFκB primary antibody was added and the ELISA plate incubated for 1 hour. Secondary antibody was added after a wash step and the plate incubated for another hour. Developing solution was added to the wells and after 45 minutes, stop solution was added, the wells washed and absorbance measured at 450 nm. The results are expressed as percentage of untreated cells.

2.3.10 Estimation of reactive oxygen species

Oxidative stress was assessed using two separate methods. The first assay selected nitrotetrazolium blue is a colorimetric assay detecting specifically superoxide however it has some limitations as it can be re-oxidised by both H₂O₂ and peroxynitrite often leading to an underestimation of superoxide production (Brandes and Janiszewski 2005). To try to overcome this and get a more general indication of reactive oxygen species generation the second assay selected utilised the fluorescent dye 2,7-dichlorodihydrofluorescein to detect overall cellular oxidative stress as it is non-specific for the radicals it detects (Brandes and Janiszewski 2005).
2.3.10.1 Nitrotetrazolium blue (NBT) assay

Cells were plated in 12 well plates and incubated until sub-confluence as previously explained (approximately 48 hours). Following the initial incubation period, the cells were treated with different concentrations of different antiretroviral drugs and nitrotetrazolium blue (NBT) added to the treatment media an incubated for a further 3-6 hours. The media was removed and 1 ml 70 % ethanol added to each well and left for 10 minutes to fix the cells. The ethanol was aspirated and the cells rinsed with 100 % methanol twice to remove any excess NBT. The cells were left to dry at room temperature. To solubilize the cells 100 μl of a 6:5 mixture of DMSO and 2 M potassium hydroxide was added to each well after drying. The solution from the different wells was transferred to individual wells in a 96 well plate and read at 705 nm. The method has been adapted from Choi et al. (Choi, Kim et al. 2006).

2.3.10.2 DCF (2,7-dichlorodihydrofluorescein) assay

Cells were plated in 96 well plates, and incubated for 24 hours prior to treatment with NNRTIs for 24 hours. The drug media was removed before addition of 100 μL 5 μM dichlorodihydrofluorescein diacetate dissolved in media (0 % FBC). The DCF solution was removed after 30 minutes incubation and the cells washed with PBS twice, then 100 μL PBS added. Dichlorodihydrofluorescein diacetate is a lipophilic substance that readily cross the cell membrane. In the cytoplasm, the compound is oxidised to the fluorescent dichlorofluorescein. The fluorescence (excitation: 485
nm, emission: 528 nM) was documented over time (30 minutes) and reflects the extent of reactive oxygen species in the cytoplasm.

### 2.3.11 Protein determination (Bradford Assay)

The Bradford assay, developed by Marion Bradford (Bradford 1976) is a colorimetric assay employed to determine the total concentrations of protein in a sample. A standard curve was prepared by using different concentrations of albumin (linearity is preserved up to 1 mg/ml albumin), see figure 2.1 below. The dye, Coomassie Brilliant Blue, is converted from red to blue in the presence of protein.

![Figure 2.1](image.png)

**Figure 2.1**  A representative protein standard curve.

200 µl Coomassie Brilliant Blue was added to 10 µl of sample with unknown protein concentration in 96 well plates and absorbance read at 595 nm. Dilution of the samples was necessary when the amount of protein exceeded that of the amount used to prepare the standard curve.
2.3.12 Western Blot

Burnette was the first to describe the Western Blot technique (Burnette 1981). This technique allows for the separation and detection of specific cellular proteins both qualitatively and quantitatively. This method was therefore chosen to determine whether antiretroviral drugs could induce ER stress in the cells of the vasculature and heart, by determining the expression levels of CHOP, a specific protein marker of ER stress (Katsoulieris, Mabley et al. 2010).

2.3.12.1 Protein extraction

EA.hy926 and H9c2 cell were cultured in 10 cm tissue culture dishes as previously explained. At approximately 80-90 % confluency the cells were treated with different concentrations of different drugs and incubated for 6-24 hours. At the end of the treatment period the media was removed from the dishes, and the cells washed twice with PBS in order to ensure all non-cell protein was removed from the plates. The cells were then transferred to 15 ml tubes and centrifuged at 1500 rpm for 5 minutes. The supernatant was removed and 150 μl RIPA buffer added to each tube in order to lyse the cells. The samples were transferred to the cold room (between 2-8 °C) for 30 minutes, and ran through a 21 G needle and syringe in order to break up any collagenous material. The samples were centrifuged for another 25 minutes in the cold room at 14680 rpm and finally the supernatant (approximately
120 μl) containing the proteins were transferred to new Eppendorf tubes and frozen at -20 °C.

2.3.12.2 Sample preparation

An equal volume of loading buffer (β-mercaptoethanol and Laemmeli sample buffer mixed in a 1:19 ratio), and sample were mixed. The resulting sample solution was boiled at 100 °C for ten minutes then incubated at 2-8 °C for ten minutes before freezing the samples at -80 °C for storage.

2.3.12.3 Separation

The combination of sodium dodecyl sulphate (SDS) polyacrylamide gel electrophoresis (PAGE) and Western blotting, the former described by Laemmli (Laemmli 1970) and latter first described by Burnette (Burnette 1981) was employed to separate and identify specific proteins in the samples, which contained a complex mixture of different proteins. Separation is based on molecular weight.

A 0.75 ml porous acrylamide gel for separation of the sample proteins was prepared in a two-step process; the separating gel was prepared and allowed to polymerise (1 hour) in glass gel plates. A 1 cm gap was left at the top of the glass plates and stacking gel added after the separating gel had polymerised. A plastic well-forming comb was inserted into the stacking gel. After 45 minutes, the glass plates were moved to the electrode assembly and placed in a tank to which 500 ml running
buffer was added. The plastic comb was removed and a molecular weight marker, the samples and a positive control where appropriate were equally loaded into individual wells in the gel. The gel was run at 100 V for 10 minutes before the voltage was increased to 110 V until the dye front reached the bottom of the gel.

2.3.12.4 Transfer
Once the run was complete (approximately 90 minutes), the gel was removed from the glass plates and added to a container containing transfer buffer. A nitrocellulose membrane, filter paper (2x) and sponges (2x) were also placed in the transfer buffer. The gel was then placed on top off the nitrocellulose membrane and packed between a layer of filter paper and sponge on either side of the nitrocellulose membrane and gel. The sandwich was placed in a gel holder cassette, and placed in a Protean tank with an ice block and magnetic stirrer. Transfer buffer was added to the Protean tank, and the power was set to 100 V for 75 minutes at 4 °C. The electrophoretic transfer of the sample proteins from the gel to the nitrocellulose membrane was first described in 1979 (Towbin, Staehelin et al. 1979).

2.3.12.5 Blocking
The sandwich was removed from the Protean tank after 75 minutes and the nitrocellulose membrane was added to a 5 % milk solution in TBS Tween for 60 minutes and left on a shaker (91 rpm). Removal of the 5 % milk solution followed.
2.3.12.6 Detection

After removal of the milk, the membrane was incubated with the primary antibody overnight (table 2.1). The secondary antibody, also prepared in 5 % milk solution, was added to the membrane the following day after washing the membrane in TBS Tween for 30 minutes (changed every 10 minutes). After a 2 hour incubation period, the membrane was washed with TBS Tween for 30 minutes (solution changed every 5 minutes) and ECL developing solution added. After 5 minutes the membrane was transferred to a hypercassette and developed using hyperfilm.

<table>
<thead>
<tr>
<th>Primary Antibody</th>
<th>Blocking solution</th>
<th>Blotting solution</th>
<th>Primary antibody solution</th>
<th>Secondary antibody solution</th>
<th>Secondary antibody dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-CHOP</td>
<td>5 % milk in TBST</td>
<td>5 % milk in TBST</td>
<td>1:2000</td>
<td>5 % milk in TBST</td>
<td>1:5000</td>
</tr>
<tr>
<td>Anti-Actin</td>
<td>5 % milk in TBST</td>
<td>5 % milk in TBST</td>
<td>1:25,000</td>
<td>5 % milk in TBST</td>
<td>1:5000</td>
</tr>
</tbody>
</table>

Table 2.1 Concentrations of antibodies and blotting/blocking solutions used in western blot experiments.

The blots were digitalised and the densitometry analysed with ImageJ. The results are expressed as density of CHOP: density of β-actin.

2.3.13 Endothelial cell function

As the major limitation of using EA.hy926 cells is their reduced capacity to produce nitric oxide another method was required to determine the effects of antiretroviral drugs on the key endothelial cell function; that of regulating vascular tone via nitric
The ability of rat endothelial cells to produce nitric oxide and relax aortic smooth muscle following exposure to acetylcholine was used to determine any effects of antiretroviral drugs on eNOS function.

Experiments with thoracic aorta from male Sprague-Dawley rats were performed to determine the effects of rilpivirine and etravirine on endothelial function. The rats were sacrificed by cervical dislocation, dissected and the thoracic portion of the aorta excised and placed in Krebs solution. Any periadventitial fat was removed and the aorta cut into 1 mm segments and placed in Hams F12 medium supplemented with 3 % FBS, endothelial cell growth supplement (0.03 mg/ml), heparin (50 U/ml) and 1 % penicillin and streptomycin. Aortic rings were treated with 1, 3 and 10 μM of rilpivirine or etravirine (with or without PJ34) and incubated for 2, 4 or 6 hours. After the incubation period each aortic ring was placed in an oxygenated isolated organ bath containing Krebs solution (10 ml) maintained at 37 °C. The apparatus was calibrated prior to each experiment. A 1.5 g constant resting tension was maintained for 1 hour, by continuous adjustments. During this period the Kreb’s solution was changed every 15 minutes. The functionality of the rings was verified by dose dependent contraction to phenylephrine (1x10^{-9} M to 1x10^{-4} M PE), followed by acetylcholine-induced (1x10^{-9} M to 1x10^{-4} M ACh) relaxation. The isometric contractions were measured and visualised with the digital software Chart version 5.2.2. The method was adapted from Helyar et al. and the results are expressed as percentage relaxation (Helyar, Patel et al. 2009).
2.4 Statistical analysis

The results are expressed as mean ± SEM, and analysed with one or two-way analysis of variance (ANOVA) in order to establish the presence or absence of statistical significance (p < 0.05, 0.01 or 0.001). Post-hoc tests: Bonferroni or Tukeys, as appropriate. Data was analysed with GraphPad Prism.
Chapter Three: The effects of efavirenz, rilpivirine and etravirine on the EA.hy926 cell line and endothelial function using rat aorta
3.1 Introduction

Efavirenz, a first generation NNRTI, in combination with emtricitabine and tenofovir, both NRTIs, is first line treatment for HIV in the UK (Williams, Churchill et al. 2014) and formulated as a once daily formulation, Atripla, to aid compliance. Etravirine was the first second generation NNRTI to be marketed (2008), and its place in the management of HIV is in virological treatment failure. Etravirine is not formulated as a once daily preparation but taken twice a day together with a boosted protease inhibitor and an agent with a third mechanism of inhibiting HIV replication (Williams, Churchill et al. 2014). Rilpivirine, the most recent second generation NNRTI to be licensed, was approved for treatment of HIV in 2011 and formulated with the same NRTIs as efavirenz: emtricitabine and tenofovir and marketed as Eviplera in the UK and Complera in the US. The second generation NNRTIs are known as diarylpyramide (DAPY) compounds and differ chemically from the first generation drugs.

Several studies have compared Atripla and Eviplera, whereas etravirine has not been studied to the same extent. Atripla and Eviplera are equally effective in terms of reducing viral load, provided the nadir viral RNA load is <100 000 copies/ml (Behrens, Rijnders et al. 2014, Cohen, Wohl et al. 2014). Efavirenz is frequently associated with psychotropic side effects, which often necessitates change of antiretroviral therapy. Rilpivirine is not associated with the same psychotropic side effects, however must be taken with a calorific meal. Furthermore rilpivirine is
associated with a better lipid profile than efavirenz (Behrens, Rijnders et al. 2014). Virological failure on the other hand is more common in patients on Eviplera compared to patients on Atripla (Behrens, Rijnders et al. 2014, Cohen, Wohl et al. 2014). Etravirine has a more favourable lipid profile than efavirenz and does not cause the same neuropsychiatric side effects, which is probably due to poor central nervous system penetration, which in turn limits its use in HIV (Schrijvers 2013).

The D:A:D study group, which identified a positive association between abacavir and myocardial infarction, could not find an association between cumulative NNRTI exposure and risk of myocardial infarction in their initial study or their follow up study (Group, Friis-Moller et al. 2007, Worm, Sabin et al. 2010) In contrast to the D:A:D study, another cohort study identified an increased risk of myocardial infarction in HIV positive patients taking efavirenz, (Durand, Sheehy et al. 2011).

The aim of the work presented in this chapter was to investigate and compare the endothelial cytotoxic effects of the first generation NNRTI efavirenz with the second generation drugs rilpivirine and etravirine. A better endothelial cell safety profile for second generation NNRTIs may translate into a better clinical outcome for HIV patients. To date no studies have been published investigating the direct cardiovascular effects of etravirine and rilpivirine.
3.2 Experimental protocols

EA.hy926 cells were treated with different concentrations (3, 10 and 30 μM) of rilpivirine, efavirenz or etravirine, with or without PARP inhibitor PJ34 (0.3, 1 and 3 μM).

The MTT assay, as described in methods section 2.3.3, was employed to measure cell viability. HPI staining and ELISA for caspase-3/7 activation were used to evaluate cell death, described in methods section 2.3.3 and 2.3.4. To determine if treatment with rilpivirine, etravirine and efavirenz lead to oxidative stress, the NBT and DCF assays were employed as described in methods section 2.3.9. SDS page and Western blotting were carried out to determine the presence of ER stress marker CHOP, as explained in the method section 2.3.11. Release of endothelin and interleukin 8 was measured with ELISAs as described in methods sections 2.3.6 and 2.3.7. Endothelial function was measured using aortic rings as described in method section 2.3.12. Lastly the activation of NFκB was determined with an ELISA kit, described in section 2.3.8.
3.3 Result

3.3.1 Effect of NNRTIs on cell viability

Cell viability was reduced in a dose and time dependent manner following treatment with all three NNRTIs in the MTT assay. Rilpivirine led to a significantly greater loss of cell viability than both efavirenz and etravirine following treatment with both 3 and 10 μM. However, efavirenz led to a greater loss of cell viability than both rilpivirine and etravirine following exposure to 30 μM. These results were seen after both 24 and 48 exposure times (figure 3.1).
Figure 3.1  Effect of increasing concentrations (3, 10 and 30 μM) of efavirenz, rilpivirine and etravirine on EA.hy926 cell viability following treatment for 24 hours (A) and 48 hours (B). Graph shows a statistically significant time and dose dependent reduction in cell viability following treatment with all NNRTIs. Data was analysed with two-way ANOVA and Bonferroni post-hoc test, and is expressed as mean ± SEM, n = 3, 6 replicates per experiment. *p<0.05  efavirenz, rilpivirine and etravirine vs. untreated control, † p<0.05 efavirenz vs. rilpivirine vs. etravirine at the same concentrations.
3.3.2 Investigation into mode of cell death

Treatment with efavirenz led to a significant increase in apoptotic and necrotic cell death, whereas treatment with etravirine and rilpivirine only led to a significant increase in necrosis when measured morphologically following HPI staining (table 3.1). Caspase-3/7 levels increased with both etravirine (10 and 30 μM) and efavirenz (30 μM) but not rilpivirine. Interestingly the increase was 3 fold higher with efavirenz (figure 3.2)
### A.

<table>
<thead>
<tr>
<th>Etravirine</th>
<th>Live</th>
<th>Apoptotic</th>
<th>Necrotic</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 μM</td>
<td>97.0 ± 1.5</td>
<td>0.4 ± 0.1</td>
<td>2.6 ± 1.6</td>
</tr>
<tr>
<td>3 μM</td>
<td>96.0 ± 1.4</td>
<td>0.2 ± 0.1</td>
<td>3.8 ± 1.4</td>
</tr>
<tr>
<td>10 μM</td>
<td>91.7 ± 3.6</td>
<td>0.3 ± 0.3</td>
<td>8.0 ± 3.8*</td>
</tr>
<tr>
<td>30 μM</td>
<td>85.4 ± 3.9</td>
<td>0.4 ± 0.1</td>
<td>14.2 ± 4.0*</td>
</tr>
</tbody>
</table>

### B.

<table>
<thead>
<tr>
<th>Rilpivirine</th>
<th>Live</th>
<th>Apoptotic</th>
<th>Necrotic</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 μM</td>
<td>98.1 ± 1.0</td>
<td>0.3 ± 0.3</td>
<td>1.7 ± 0.7</td>
</tr>
<tr>
<td>3 μM</td>
<td>97.9 ± 0.6</td>
<td>0.1 ± 0.1</td>
<td>2.0 ± 0.5</td>
</tr>
<tr>
<td>10 μM</td>
<td>97.1 ± 0.8</td>
<td>0.1 ± 0.1</td>
<td>2.9 ± 0.7</td>
</tr>
<tr>
<td>30 μM</td>
<td>93.1 ± 0.5</td>
<td>0.2 ± 0.1</td>
<td>6.7 ± 0.4*</td>
</tr>
</tbody>
</table>

### C.

<table>
<thead>
<tr>
<th>Efavirenz</th>
<th>Live</th>
<th>Apoptotic</th>
<th>Necrotic</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 μM</td>
<td>98.8 ± 0.2</td>
<td>0.10 ± 0.1</td>
<td>1.0 ± 0.1</td>
</tr>
<tr>
<td>3 μM</td>
<td>98.2 ± 0.4</td>
<td>0.38 ± 0.2</td>
<td>1.4 ± 0.2</td>
</tr>
<tr>
<td>10 μM</td>
<td>97.7 ± 0.2</td>
<td>0.17 ± 0.1</td>
<td>2.1 ± 0.2*</td>
</tr>
<tr>
<td>30 μM</td>
<td>82.5 ± 4.5</td>
<td>3.4 ± 1.0*</td>
<td>14.1 ± 4.3*</td>
</tr>
</tbody>
</table>

Table 3.1  Quantification of apoptosis and necrosis by HPI staining of EA.hy926 cells following 24 hours treatment with increasing concentrations (3, 10 and 30 μM) of etravirine (A), rilpivirine (B) and efavirenz (C). Data was analysed with two-way ANOVAs and Bonferroni post-hoc test, and is expressed as mean percentage of 100 % ± SEM, n = 3-5. *p<0.05 etravirine, rilpivirine and efavirenz vs. untreated control.
Figure 3.2  Quantification of caspase-3/7 following 24 hours treatment with increasing concentrations (3, 10 and 30 μM) of efavirenz, rilpivirine and etravirine. Etravirine and efavirenz, but not rilpivirine increased caspase-3/7 activity. Data was analysed with two-way ANOVA and Bonferronis post-hoc test and is expressed as mean percentage of control ± SEM, n = 3-4. ***p<0.001 and * p<0.05 etravirine or efavirenz vs. untreated control.

3.3.3 Oxidative stress determination

Oxidative stress, measured with DCF, did not increase significantly following treatment with any of the NNRTIs (table 3.2), however, a decrease in fluorescence was observed with the highest concentration of efavirenz. DCF was calculated according to the following formula: (Ft₂₈ - Ft₀) x 100 giving percentage change in fluorescence, where Ft₂₈ is fluorescence at 28 minutes and Ft₀ is fluorescence at 0 minutes. These results were confirmed by the NBT assay, in which treatment with efavirenz and rilpivirine did not lead to a significant increase in superoxide production (table 3.3).
Table 3.2  Change in oxidative stress measured by DCF following 24 hours treatment with etravirine, rilpivirine and etravirine measured over a 28-minute period. Data was analysed with two-way ANOVA and expressed as mean percentage of untreated cells ± SEM, n = 5, *p<0.05 efavirenz vs. untreated control.

<table>
<thead>
<tr>
<th></th>
<th>0 μM</th>
<th>3 μM</th>
<th>10 μM</th>
<th>30 μM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Etravirine</td>
<td>100 ± 2</td>
<td>111 ± 8</td>
<td>93 ± 5</td>
<td>81 ± 7</td>
</tr>
<tr>
<td>Rilpivirine</td>
<td>100 ± 3</td>
<td>108 ± 11</td>
<td>93 ± 15</td>
<td>87 ± 15</td>
</tr>
<tr>
<td>Efavirenz</td>
<td>100 ± 1</td>
<td>89 ± 5</td>
<td>98 ± 5</td>
<td>45 ± 14*</td>
</tr>
</tbody>
</table>

Table 3.3  Change in superoxide levels as a marker of oxidative stress following treatment with rilpivirine and efavirenz (3 - 30 μM). Treatment with efavirenz and rilpivirine did not significantly increase oxidative stress. Data was analysed with two-way ANOVA and expressed as percentage of untreated cells (mean± SEM), n=2, 3 replicates per experiment.

<table>
<thead>
<tr>
<th></th>
<th>0 μM</th>
<th>3 μM</th>
<th>10 μM</th>
<th>30 μM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rilpivirine</td>
<td>100 ±4.2</td>
<td>104 ± 5.3</td>
<td>102 ± 4.5</td>
<td>101 ± 2.0</td>
</tr>
<tr>
<td>Efavirenz</td>
<td>100 ±5.2</td>
<td>100 ± 2.5</td>
<td>115 ± 13</td>
<td>105 ± 6.5</td>
</tr>
</tbody>
</table>

3.3.4 Measurements of CHOP, a marker of ER stress

Western blot performed on proteins extracted following 24 hours treatment with increasing concentrations of rilpivirine, etravirine and efavirenz (3, 10 and 30 μM) showed that none of the NNRTIs increased the expression of ER stress marker protein CHOP (figure 3.3).
Figure 3.3 Representative blot of proteins extracted from EA.hy926 cells following treatment with increasing concentrations of rilpivirine, etravirine and efavirenz (3 – 30 µM) for 24 hours. The NNRTIs did not induce ER stress as demonstrated by no increase in the expression of ER stress marker CHOP (top) between treated and untreated cells. Tunicamycin (T) and thapsigargin (Th) were used as positive controls. N = 2.

3.3.5 Measurements of interleukin 8 secretion as a marker of inflammation

Treatment of endothelial cells with etravirine for 24 hours led to a large increase in interleukin 8 (IL-8) secretion by the EA.hy926 cells (figure 3.4). A 70x increase in IL-8 secretion was seen in the cells treated with 30 µM etravirine vs. untreated cells. A significant increase in IL-8 secretion was also seen following treatment with both 30 µM rilpivirine and 30 µM efavirenz (figure 3.4). Using 10 µM etravirine and 30 µM of both efavirenz and rilpivirine a time profile of IL-8 release was determined (figure 3.5). There was no increase in IL-8 with any of the drugs following 2, 4 or 6 hours of exposure but IL-8 release was significantly increased at 12 and 24 hours for all three drugs.
Figure 3.4  Increase in IL-8 levels in response to exposure to increasing concentrations of efavirenz, rilpivirine and etravirine for 24 hours. Graph shows a significant increase in IL-8 following treatment with both 10 and 30 µM etravirine, but only with 30 µM rilpivirine and efavirenz. Data was analysed with two-way ANOVA and Bonferroni post-hoc test and are expressed as mean ± SEM, n=3-4, 3 replicates per experiment. ***p<0.001 and *p<0.05 etravirine, rilpivirine or efavirenz vs. untreated control, ††† p<0.001 etravirine 10 µM vs. etravirine 30 µM.
Figure 3.5  Increase in IL-8 in response to 30 μM efavirenz, 30 μM rilpivirine and 10 μM etravirine over time (2 – 24 hours). Graph shows a significant increase in IL-8 following 12 and 24 hours treatment for all NNRTIs. Data was analysed with two-way ANOVA and Bonferroni post-hoc test and are expressed as mean ± SEM, n=3, 3 replicates per experiment. ***p<0.001 and *p<0.05 etravirine, rilpivirine or efavirenz vs. untreated control.

3.3.6 Effect of NNRTIs on endothelin secretion

A trend towards an increase was seen in endothelin secretion following treatment with 3 μM efavirenz, although this was not statistically significant. A decreased level of endothelin was however seen following treatment with all concentrations of etravirine and 10 and 30 μM rilpivirine and efavirenz (table 3.4).
Table 3.4  Effect of etravirine, rilpivirine and efavirenz on endothelin release from EA.hy926 cells measured by ELISA. There was a decrease in endothelin following treatment with the NNRTIs. Data was analysed with two-way ANOVA and is expressed as mean percentage of untreated cells, n =3. *p<0.05 etravirine, rilpivirine and efavirenz vs. untreated control.

<table>
<thead>
<tr>
<th></th>
<th>0 µM</th>
<th>3 µM</th>
<th>10 µM</th>
<th>30 µM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Etravirine</td>
<td>100 ± 1.8</td>
<td>82.2 ± 4.8*</td>
<td>19.2 ± 1.7*</td>
<td>8.1 ± 1.9*</td>
</tr>
<tr>
<td>Rilpivirine</td>
<td>100 ± 2.2</td>
<td>99.5 ± 1.8</td>
<td>50.4 ± 21.5*</td>
<td>41.5 ± 4.6*</td>
</tr>
<tr>
<td>Efavirenz</td>
<td>100 ± 3.5</td>
<td>114 ± 5.4</td>
<td>82 ± 2.7*</td>
<td>4.2 ± 1.7*</td>
</tr>
</tbody>
</table>

3.3.7 NFκB activation

An increase in activated NFκB (Nuclear factor kappa-light-chain-enhancer of activated B) levels was seen after treatment with all NNRTIs (figure 3.6). The timed profile of NFκB activation differed between the NNRTIs. Rilpivirine activated NFκB levels after 1 hour treatment, efavirenz after 2 hours and etravirine activated NFκB at all time points (1, 2 and 4 hours, figure 3.6).
Figure 3.6  Effect of 30 μM efavirenz, rilpivirine and etravirine on activation of transcription factor NFκB. Treatment with all NNRTIs led to an increase in NFκB. Data was analysed with two-way ANOVA and Bonferroni post-hoc test and is expressed as mean percentage ± SEM, n=3-4, 2 replicates per experiment. *p<0.05 and **p<0.01 untreated vs. treated cells.

3.3.8 Endothelial function measured with rat aortic rings

A dose and time dependent decrease in endothelial cell function was seen following treatment of rat aortic rings with both etravirine and rilpivirine (1, 3 and 10 μM), as demonstrated by EC50 values (table 3.5). The decrease in endothelial function was statistically significant following 4 and 6h treatment with both NNRTIs (figure 3.7).
Table 3.5  EC$_{50}$ of acetylcholine response following exposure of ex vivo thoracic aortic rings from Sprague–Dawley rats to rilpivirine and etravirine for 2, 4 and 6 hours. Both NNRTIs increased the EC$_{50}$ values. Data is expressed as mean ± SEM from 12 animals.
Figure 3.7  Effect of etravirine and rilpivirine on endothelial function in rat aortic rings. Rat aortic rings were exposed to increasing concentrations of etravirine or rilpivirine for 2 hours (A), 4 hours (B) and 6 hours (C). Data was analysed with two-way ANOVA with Bonferroni correction and is expressed as mean ± SEM, n=14, *p<0.05 etravirine or rilpivirine vs. untreated control.
3.3.9 Involvement of PARP in NNRTI damage

3.3.9.1 Effect of PARP inhibitor PJ34 on NNRTI induced loss of cell viability and mode of cell death

Inhibition of PARP with PJ34 failed to protect against the loss of cell viability observed with rilpivirine, etravirine and efavirenz (table 3.6). PARP inhibition did lead to a trend towards a decrease in apoptosis in efavirenz treated cells when measured morphologically after HPI staining, however statistical significance was not reached. The PARP inhibitor did not reduce the NNRTI-mediated increase in necrosis levels (table 3.7).
<table>
<thead>
<tr>
<th></th>
<th>0 μM</th>
<th>0.3 μM</th>
<th>1 μM</th>
<th>3 μM</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Rilpivirine</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10 μM</td>
<td>71 ± 1.4*</td>
<td>74 ± 2.1</td>
<td>71 ± 2.1</td>
<td>70 ± 1.5</td>
</tr>
<tr>
<td>30 μM</td>
<td>71 ± 1.6*</td>
<td>69 ± 1.6</td>
<td>69 ± 1.3</td>
<td>69 ± 1.0</td>
</tr>
<tr>
<td><strong>Etravirine</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10 μM</td>
<td>70 ± 1.5*</td>
<td>70 ± 1.4</td>
<td>72 ± 1.3</td>
<td>73 ± 1.2</td>
</tr>
<tr>
<td>30 μM</td>
<td>55 ± 1.2*</td>
<td>55 ± 1.3</td>
<td>57 ± 1.2</td>
<td>59 ± 1.6</td>
</tr>
<tr>
<td><strong>Efavirenz</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10 μM</td>
<td>101 ± 3.4</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>30 μM</td>
<td>67 ± 3.7*</td>
<td>63 ± 3.7</td>
<td>67 ± 3.5</td>
<td>64 ± 0.8</td>
</tr>
</tbody>
</table>

Table 3.6  Cell viability results following simultaneous treatment with NNRTIs and PJ34 for 24 hours. Treatment with PJ34 in combination with the NNRTIs did not improve loss of cell viability caused by the NNRTIs. Data is expressed as mean percentage of untreated cells ± SEM, and was analysed with three, two-way ANOVAs, n = 2, 6 replicates per experiment. *p<0.05 cells treated with NNRTI vs. untreated cells.
A. Live | Apoptotic | Necrotic
--- | --- | ---
0 μM | 96.86 ± 1.18 | 0.44 ± 0.15 | 2.70 ± 1.14
30 μM rilpivirine | 83.52 ± 2.37 | 0.47 ± 0.24 | 16.01 ± 2.26*
30 μM rilpivirine and 3 μM PJ34 | 84.50 ± 3.6 | 0.67 ± 0.3 | 14.84 ± 3.55

B. Live | Apoptotic | Necrotic
--- | --- | ---
0 μM | 97.82 ± 0.29 | 0.37 ± 0.15 | 1.81 ± 0.26
30 μM etravirine | 76.45 ± 3.76 | 0.45 ± 0.18 | 23.10 ± 3.91*
30 μM etravirine and 3 μM PJ34 | 77.14 ± 6.07 | 0.05 ± 0.05 | 22.81 ± 0.05

C. Live | Apoptotic | Necrotic
--- | --- | ---
0 μM | 98.09 ± 0.21 | 0.49 ± 0.16 | 1.42 ± 0.15
30 μM efavirenz | 80.32 ± 1.99 | 4.91 ± 1.37* | 14.77 ± 2.57*
30 μM efavirenz and 3 μM PJ34 | 84.32 ± 4.34 | 1.80 ± 0.52* | 13.88 ± 4.84

Table 3.7  Effect of PARP inhibitor PJ34 on apoptosis and necrosis following 24 hours treatment with 30 μM rilpivirine (A), etravirine (B) and efavirenz (C) in combination with 3 μM PJ34. There was a significant decrease in apoptosis in cells treated with efavirenz together with PJ34. Necrotic cell death remained unchanged. Data was analysed with two-way ANOVAs and Bonferroni's post-hoc test and is expressed as mean percentage of 100 % ± SEM, n = 3. *p<0.05 cells treated with NNRTI vs. untreated cells.
3.3.9.2 Effect of PARP inhibition on NNRTI-mediated increase in interleukin 8 release

There was a trend towards decreased levels of IL-8 following PARP inhibition in efavirenz treated cells, however PJ34 did not reduce the amount of IL-8 secreted following treatment with rilpivirine nor etravirine (table 3.8).

<table>
<thead>
<tr>
<th></th>
<th>PJ34</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0 μM</td>
</tr>
<tr>
<td>Untreated</td>
<td>1.4 ± 0.14</td>
</tr>
<tr>
<td>30 μM efavirenz</td>
<td>10.8 ± 2.3*</td>
</tr>
<tr>
<td>30 μM rilpivirine</td>
<td>11.8 ± 1.2*</td>
</tr>
<tr>
<td>10 μM etravirine</td>
<td>27.2 ± 2.1*</td>
</tr>
</tbody>
</table>

Table 3.8 IL-8 release following treatment of EA.hy926 cells with NNRTIs with and without PJ34. The amount of IL-8 released was not significantly different following treatment between NNRTIs and NNRTIs in combination with PJ34, although there was a trend towards a decrease with efavirenz and PJ34, although this did not reach statistical significance. Data was analysed with three one-way ANOVAs, n=3. *p<0.05 cells treated with NNRTI vs. untreated cells.

3.3.9.3 Involvement of PARP in NNRTI induced loss of endothelial function

The decrease in endothelial function seen following treatment of rat aortic rings with 10 μM etravirine and rilpivirine, was reversed with 3 μM PJ34, as demonstrated by EC<sub>50</sub> values (table 3.9). The improvement in endothelial function was statistically significant for both etravirine and rilpivirine (figure 3.8).
Table 3.9  The EC50 of acetylcholine in response to exposure of *ex vivo* thoracic aortic rings from Sprague–Dawley rats to 10 μM rilpivirine or etravirine and 3 μM PARP inhibitor PJ34 for 4 hours. PARP inhibition reduced the EC50 values. Data is expressed as mean ± SEM from 12 animals. The EC50 values of acetylcholine in aortic rings treated with increasing concentrations of etravirine and rilpivirine in combination with PJ34.

<table>
<thead>
<tr>
<th></th>
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<th>10 μM etravirine</th>
<th>10 μM etravirine and 3 μM PJ34</th>
</tr>
</thead>
<tbody>
<tr>
<td>EC50 (nM)</td>
<td>31.2 ± 5</td>
<td>134.0 ± 30</td>
<td>91.7 ± 13</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>Untreated</th>
<th>10 μM rilpivirine</th>
<th>10 μM rilpivirine and 3 μM PJ34</th>
</tr>
</thead>
<tbody>
<tr>
<td>EC50 (nM)</td>
<td>28.8 ± 3</td>
<td>105.3 ± 10</td>
<td>54.2 ± 7</td>
</tr>
</tbody>
</table>
Figure 3.8  Involvement of PARP in NNRTI mediated endothelial dysfunction in rat aortic rings. Rat aortic rings were exposed to 10 μM rilpivirine (A) or 10 μM etravirine (B) with or without 3μM of the PARP inhibitor PJ34 for four hours. PJ34 significantly improved NNRTI mediated endothelial dysfunction. Data was analysed with two way ANOVA and is expressed as mean ± SEM, n=13, *p<0.05 rilpivirine or etravirine vs. untreated control.
3.4 Discussion

The data presented confirm the results previously obtained in our lab in which treatment with efavirenz decreased endothelial cell viability, increased apoptosis and necrosis and led to endothelial dysfunction (Faltz 2013). We have expanded upon this demonstrating that apoptosis induced by efavirenz is caspase dependent. Furthermore, we have also shown that efavirenz decreased endothelin secretion and increased secretion of the pro-inflammatory marker interleukin 8, the latter possibly mediated by an increase in NFκB activation. Following on from this, we are the first to show that the second generation NNRTIs rilpivirine and etravirine have similar damaging effects on the endothelial cells to the first generation drug efavirenz.

All three NNRTIs led to a dose and time dependent reduction in cell viability in the MTT assay. Interestingly treatment with rilpivirine led to a greater loss of cell viability in comparison to efavirenz following exposure to 10 μM whereas treatment with 30 μM had the opposite effect: efavirenz led to a greater loss of cell viability than both etravirine and rilpivirine, indicating different underlying mechanisms. The explanation may be related to the chemical structures; etravirine and rilpivirine are both diarylpyrimidine (DAPY) compounds, differing structurally to efavirenz. Whereas efavirenz has been shown to cause a decrease in cell viability in human umbilical vein endothelial cells (Apostolova, Gomez-Sucerquia et al. 2010), no publications have to date evaluated the cell viability effects of rilpivirine and
etravirine on any endothelial cell line. The reduction in cell viability may be due to a reduction in cell number but can also be caused by reduced mitochondrial activity. Indeed, the loss of cell viability is associated with a loss of mitochondrial function (Brand and Nicholls 2011).

NRTIs such as zidovudine and stavudine have been shown to inhibit DNA polymerase-γ and subsequent oxidative stress cause loss of cell viability (Kakuda 2000). NNRTIs like efavirenz, rilpivirine and etravirine are non-competitive inhibitors of HIV reverse transcriptase and do not inhibit DNA polymerase-γ and were proposed to be less likely to have mitochondrial toxic effects and therefore less likely to increase cellular oxidative stress and decrease cell viability. However, previous work from our group demonstrated that efavirenz decreased endothelial cell viability (confirmed here) and increased cellular oxidative stress likely through disruption of mitochondrial function. Indeed, efavirenz has been shown by another group to increase endothelial cell oxidative stress via disruption of endothelial mitochondrial membrane potential leading to a loss of cell viability (Jamaluddin, Lin et al. 2010). This supports other published work that has shown efavirenz in vitro can increase cellular oxidative stress in a variety of other cell types including hepatocytes where it was again found to have mitochondrial toxic effects inhibiting mitochondrial complex I (Blas-Garcia, Apostolova et al. 2010). Protective actions of antioxidants against efavirenz-mediated damage in both endothelial and hepatic cells have also been observed implicating mitochondrial-generated oxidative stress
as a key mediator of cell dysfunction (Apostolova, Gomez-Sucerquia et al. 2010, Jamaluddin, Lin et al. 2010).

Though we were unable to detect an increase in endothelial cell oxidative stress following exposure to efavirenz, rilpivirine and etravirine using the NBT and DCF assays this may be because of a lack of sensitivity of these assays rather than no increase in oxidative stress and maybe using a specific assay to measure mitochondrial oxidative stress such as MitoSOX would have given us a different result. It is tempting to hypothesize that the decrease in endothelial cell viability/mitochondrial function with second generation NNRTIs may be related to mitochondrial toxicity and increased oxidative stress. Therefore measuring mitochondrial membrane potential in endothelial cells exposed to etravirine and rilpivirine may reveal more about the mechanisms by which these drugs are causing a loss of cell viability and possibly increasing cellular oxidative stress. Similarly, measuring the specific activity of mitochondrial complex I and maybe also II-IV following exposure to these second generation NNRTIs would be valuable in understanding the mechanism behind the loss of endothelial cell viability.

In clinical studies efavirenz has been shown to increase oxidative stress in patients treated with HAART, measured by plasma F2 isoprostane concentrations (Hulgan, Morrow et al. 2003). These studies have yet to be repeated in patients being treated
with rilpivirine or etravirine but as the number of patients on these drugs increase it may be interesting to determine if the same increase in plasma $F_2$ isoprostane concentration is observed.

Treatment with all three NNRTIs increased necrosis levels in endothelial cells. Although traditionally, necrosis has been associated with unregulated cell death, more recently it has been shown that it can be a highly regulated process (Golstein and Kroemer 2007). Pathways through which necrosis can occur include DNA-damage which can lead to PARP overactivation, which in turn leads to ATP-depletion and necrosis (Ha and Snyder 1999). Increased cellular production of reactive oxygen species, increased cytosolic calcium concentrations and activation of death receptors are other pathways leading to necrosis (Kung, Konstantinidis et al. 2011) and it may be that one or a combination of these pathways is involved in NNRTI mediated necrosis.

There was a significant increase in apoptosis following treatment with efavirenz in the above experiments. Exposure to rilpivirine and etravirine did not lead to an increase in apoptosis. Apoptosis can result from both a caspase-dependent or caspase-independent pathway (Hongmei 2012). Movement of cytochrome C and/or small mitochondrial derived activators of caspase (SMAC) from the mitochondria initiate the caspase-dependent pathway and result in the activation of caspase-3/7, which lead to oligonucleosomal DNA fragmentation. On the other hand, a well
characterized caspase-independent pathway involves the movement of apoptosis-inducing factor (AIF) from the mitochondria, which leads to large scale DNA fragmentation and apoptotic cell death (Hongmei 2012). Interestingly a significant five-fold increase in caspase-3/7 activation was measured after treatment with 30 μM efavirenz, confirming the apoptotic stimulating effects of efavirenz, through the caspase-dependent pathway. Efavirenz was first shown to increase apoptosis in Jurkat cells, with a decrease in mitochondrial transmembrane potential, release of cytochrome C and activation of caspase (Pilon, Lum et al. 2002). In hepatocytes, efavirenz has also been shown to trigger apoptosis through the intrinsic pathway (Apostolova, Gomez-Sucerquia et al. 2010).

Apoptosis can result from ER stress, which in turn is associated with the development of atherosclerosis (Tabas 2010). Tunicamycin, an inducer of ER stress, has been shown to increase the expression of chemokines involved in attracting monocytes to the endothelium as well as chemokines involved in the monocyte transmigration across the endothelium in an ER stress dependent manner in human aortic endothelial cells (Gargalovic, Gharavi et al. 2006). In the above experiments however, the NNRTIs did not induced the expression of the ER stress marker CHOP, suggesting ER stress is not a trigger for the observed apoptosis with etravirine and rilpivirine.
Although treatment with the NNRTIs did not lead to ER stress, they all increased the release of the inflammatory chemokine interleukin 8 (IL-8). Interestingly following treatment with 30 μM etravirine, a particularly large increase in IL-8 was seen, which increased the levels 70 fold in comparison to untreated control and 20 fold when compared to rilpivirine and efavirenz. This is the first time NNRTIs have been shown to cause an increase in IL-8 levels. The involvement of IL-8 in cardiovascular diseases is supported by both in vitro and in vivo (Gargalovic, Gharavi et al. 2006). Specifically, in the endothelium, the role of IL-8 is involved in the adhesion of monocytes to the endothelium. This was first reported in human umbilical vein endothelial cells in a study published in Nature in 1999, and is recognised as an early event in the formation of atheroma (Gerszten, Garcia-Zepeda et al. 1999). Efavirenz has previously been shown to increase the adhesion of leukocytes to the endothelium, but through an effect on the leukocytes rather than the endothelial cells (Orden, De Pablo et al. 2014).

The NNRTI-mediated increase in IL-8 release may be explained by three possible mechanisms, firstly these drugs may be stimulating release of IL-8 already stored within the endothelial cell, secondly they may be increasing transcription of IL-8 or thirdly they may be increasing translation of IL-8 mRNA to protein. In the time-profile experiments, there was no increase in IL-8 secretion until after approximately 12 hour exposure to the NNRTIs, suggesting that the increased transcription or translation hypothesis is correct rather than the increase in release
of IL-8 from cellular stores. The time lag of IL-8 release may indicate a requirement for mRNA to be synthesized or increased protein synthesis before an increase in IL-8 release is observed. Expression of interleukin 8 is regulated through a signalling pathway involving mitogen activated protein kinases (MAPKs) and NFkB (Hoffmann, Dittrich-Breiholz et al. 2002). Indeed, when measured, the levels of NFkB were raised following treatment with all three NNRTIs suggesting a transcriptional mechanism of increasing IL-8 release is likely though an effect on translation cannot be ruled out. Interestingly the NFkB activation profiles differed between the different NNRTIs: etravirine was the only NNRTI which sustained significantly higher levels at all time points (1, 2 and 4 hours). The prolonged activation of NFkB may explain why secreted interleukin 8 levels were significantly higher with etravirine than the other two NNRTIs. It would have been interesting to determine if all NNRTIs activate the different MAPK pathways to the same extent, as e.g. increased activation of the p38 MAPK could also explain the difference in secretion levels between the different NNRTIs based on post-transcriptional stabilisation of IL-8 mRNA (Hoffmann, Dittrich-Breiholz et al. 2002). Furthermore, future work could include measurements of mRNA IL-8 levels using rtPCR to confirm the results obtained in the above ELISA and confirm the increased expression hypothesis.

Endothelin, a potent vasoconstrictor, was recognised over 25 years ago (Yanagisawa, Kurihara et al. 1988). It is synthesised in endothelial cells and binds to
ETA receptors on vascular smooth muscle where it activates several signalling pathways that lead to an increase in cytosolic calcium which results in vascular smooth muscle contraction (Lovenberg and Miller 1990). An increase in endothelin is associated with cardiovascular diseases (Kolettis, Barton et al. 2013). NRTIs have been shown to increase endothelin levels in human umbilical vein endothelial cells (Xue, Hebert et al. 2013) as well as increase circulating levels in rats (Jiang, Hebert et al. 2006). However, in the above experiments however, a decrease in endothelin levels was seen following treatment with 10 and 30 µM of all three NNRTIs. Interestingly, there was a trend towards an increase in endothelin following treatment with 3 µM efavirenz. In the in vitro experiments performed by Xue et al. 5 µM of the NRTIs were used, indicating that perhaps the concentrations chosen in the above experiments were too high. It would be interesting to do a release profile over time with lower concentrations of NNRTIs as they may induce a biphasic release pattern increasing endothelin release at low concentrations while inhibiting at higher concentrations. Measuring mRNA levels of endothelin following NNRTI exposure may also reveal possible cellular pathological processes that may contribute to an increased risk of vasoconstriction and hence hypertension.

Endothelial dysfunction is an early event in the progression of atherosclerosis. NRTIs and PIs have both been shown to reduce endothelium-dependent relaxation in mice and porcine coronary arteries (Sutliff, Dikalov et al. 2002, Chai, Yang et al. 2005, Jiang, Khandelwal et al. 2010). In the above experiments, treatment with both
etravirine and rilpivirine resulted in a significant dose and time dependent reduction in endothelium-dependent relaxation. This mirrors the results we have previously obtained with efavirenz in our lab (Faltz 2013). *In vivo*, efavirenz based HAART has been shown to lead to a worsening in endothelial function measured by flow mediated dilation (FMD) in comparison to PI based regimes (Gupta, Shen et al. 2012). A small pilot study in human volunteers showed that 28 days exposure to etravirine did not increase FMD contrasting the *ex vivo* results obtained in the rat aortic rings above (Gupta, Mi et al. 2011), though it is worth noting antiretroviral treatment is life long, and a longer treatment period might have given a different result.

Previous work in our lab has shown that efavirenz causes endothelial cell dysfunction mediated by overactivation of PARP (unpublished data). Overactivation of PARP in endothelial cells leads to impairment of blood vessel relaxation (Soriano, Virag et al. 2001) by a mechanism involving cellular depletion of NAD levels and subsequent depletion of the eNOS co-factor NADPH, resulting in suppression of eNOS activity and NO production (Soriano, Pacher et al. 2001). Etravirine and rilpivirine also caused endothelial cell dysfunction, and similarly to efavirenz, protection was seen with PARP inhibition suggesting that cellular depletion of NADPH and suppression of eNOS activity may be partially responsible for the loss of function observed with rilpivirine and etravirine exposure. However, as for efavirenz the EC50 values for acetylcholine-mediated NO-induced relaxation in aortic
rings exposed to rilpivirine and etravirine were not returned to those observed in untreated rings by the PARP inhibitor PJ34, suggesting PARP activation may not be the only mechanism by which NNRTIs induce endothelial cell dysfunction. Etravirine and rilpivirine could possibly scavenge NO possibly by producing ROS, inhibit eNOS directly or interfere with the production of one of the other co-factors required for NO synthesis e.g. tetrahydrobiopterin or flavin mononucleotide. This could be addressed in future studies in primary human endothelial cells. We were unable to determine the effect of these drugs on NO production using the EAhy.926 cells as these cells have a limited ability to produce nitric oxide.

PARP activation has been shown to mediate both necrosis and apoptosis. Depletion of cellular levels of NAD and high-energy phosphates by overactivation of PARP has been shown to result in cellular necrosis. By inhibiting PARP, cellular pools of NAD and ATP are not depleted and cellular necrosis can be prevented. Interestingly inhibition of PARP failed to protect against the increased necrosis levels observed with all three of the NNRTIs suggesting that the other pathways of inducing cellular necrosis mentioned earlier may be mediating endothelial cell necrosis rather than activation of PARP.

PARP activation has also been shown to mediate apoptosis through regulating the release of apoptotic proteins from the mitochondria, either directly by increasing the release of cytochrome C or indirectly via the PAR polymer mediating the release
of apoptosis inducing factor (AIF). AIF is a caspase-independent cell death effector that triggers chromatin condensation and DNA degradation leading to apoptosis. In addition, AIF has recently been identified as playing a vital mitochondrial role in healthy cells. A segment of AIF has been shown to carry an NADH-oxidase domain that regulates complex I of the respiratory chain. Efavirenz has previously been shown to inhibit the activity of mitochondrial complex I (Blas-Garcia, Apostolova et al. 2010) and it may be this is mediated through a PARP-dependent release of AIF impairing its activity. Cytochrome C release from the mitochondria activates a caspase-dependent apoptotic pathway activating caspase 9, which then subsequently activates caspase 3 and 7 to induce apoptosis. PARP inhibition has been shown to reduce cytochrome C release from mitochondria and hence inhibit this apoptotic pathway (Chen, Zsengeller et al. 2004). PARP inhibition was able to partially protect against efavirenz-mediated apoptosis suggesting that one or all of the above pathways plays a role in this efavirenz-mediated effect.

PARP activation has also been linked to increased activation of transcription factor NFκB (Kameoka, Ota et al. 2000). Inhibition of PARP showed a trend towards a decrease in efavirenz mediated IL-8 release, however IL-8 release following treatment with rilpivirine and etravirine was unaffected by PARP inhibition. These results suggest PARP overactivation, and therefore PARP mediated NFκB activation may not involved in the mechanism leading to increases in IL-8 release. We would have to confirm that the PARP inhibitor did have an effect on NFκB activation by the
NNRTIs. In previous work 3 μM PJ34 has been shown to be effective at reducing stimulated PARP activity by over 90% in EA.hy926 cells (Faltz 2013).

In conclusion, treatment with both etravirine and rilpivirine resulted in endothelial cell dysfunction including loss of NO production and increase in inflammatory cytokine release. The results presented show that the second generation NNRTIs etravirine and rilpivirine have similar damaging effects on endothelial cell function as the first generation NNRTI efavirenz, although to a lesser extent, suggesting that they may have a safer cardiovascular side effect profile.
Chapter Four: The effects of efavirenz, rilpivirine and etravirine on the myocardial H9c2 cell line
4.1 Introduction

Efavirenz, the most commonly prescribed NNRTI in the Western world, has been associated with cardiovascular complications in several clinical studies (Durand, Sheehy et al. 2011, Maggi, Bellacosa et al. 2011), whereas the studies evaluating the cardiovascular effects of the newer, less commonly prescribed NNRTIs rilpivirine and etravirine are lacking.

On a cellular level, efavirenz has been shown to cause loss of cell viability, an increase apoptosis and necrosis and ER stress in H9c2 cells by our research group (Faltz 2013). These direct toxic effects on cardiac may be the underlying cause of the increased risk of cardiovascular complications seen in HIV patients on efavirenz. The aim of the work presented in this chapter was to investigate and compare the cytotoxic effects of the first generation efavirenz with second generation rilpivirine and etravirine on the H9c2 cell line and determine if the cell effects of the second generation NNRTIs could be caused by ER stress and/or oxidative stress and therefore establish if the second generation drugs have a better cardiovascular side effect profile than the first generation drugs. To date no studies have been published investigating the cardiovascular effects of etravirine and rilpivirine on a cellular level.
4.2 Experimental protocols

H9c2 cells were treated with different concentrations (3, 10 and 30 μM) of rilpivirine, efavirenz or etravirine, with or without PARP inhibitor PJ34 (0.3, 1 and 3 μM) and the PERK inhibitor GSK 2606414 (0.1, 0.3 and 1 μM).

The MTT assay, as described in methods section 2.3.3, was employed to measure cell viability. HPI staining, ELISA assays for caspase-3/7 activation and cytoplasmic histone-associated-DNA-fragments as a marker of apoptotic cell death were used to evaluate cell death, described in methods section 2.3.3, 2.3.4 and 2.3.5. To determine if treatment of rilpivirine, etravirine and efavirenz led to the production of superoxide, the NBT and DCF assays were employed as described in methods section 2.3.9. SDS page and Western blotting were carried out to determine the presence of ER stress marker CHOP, as explained in the method section 2.3.11.
4.3 Results

4.3.1 Effect of NNRTIs on cell viability

Treatment with etravirine, rilpivirine and efavirenz led to a dose and time dependent decrease in cell viability. Interestingly at 10 μM, rilpivirine and etravirine led to a statistically significant decrease in cell viability as compared to efavirenz, whereas treatment with 30 μM had the opposite effect; efavirenz exposure led to a greater decrease in loss of cell viability than exposure to rilpivirine and etravirine following 24 hours treatment (figure 4.1 A and B).
Figure 4.1  Effect of increasing concentrations (3, 10 and 30 μM) of efavirenz, rilpivirine and etravirine on H9c2 cell viability following treatment for 24 hours (A) and 48 hours (B). Graph shows a statistically significant time and dose dependent reduction in cell viability following treatment with all NNRTIs. Data was analysed with two-way ANOVA and Bonferroni post-hoc test, and is expressed as mean ± SEM, \( n = 3 \), 6 replicates per experiment. *\( p<0.05 \) etravirine, rilpivirine and efavirenz vs. untreated control, † \( p<0.05 \) etravirine vs. rilpivirine vs. efavirenz at the same concentrations.
4.3.2 Investigation into mode of cell death

4.3.2.1 Morphological analysis of apoptosis and necrosis

Treatment with efavirenz led to cell death both via the apoptotic and necrotic pathway, whereas treatment with rilpivirine and etravirine only resulted in necrotic cell death. The increase in necrosis was statistically significant following treatment with 10 and 30 µM of all three NNRTIs (table 4.1).
<table>
<thead>
<tr>
<th></th>
<th>Alive</th>
<th>Apoptotic</th>
<th>Necrotic</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Etravirine</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0 μM</td>
<td>98.3 ± 0.5</td>
<td>0.1 ± 0.1</td>
<td>1.6 ± 0.4</td>
</tr>
<tr>
<td>3 μM</td>
<td>95.4 ± 2.6</td>
<td>0.1 ± 0.1</td>
<td>4.5 ± 2.6</td>
</tr>
<tr>
<td>10 μM</td>
<td>90.0 ± 2.1</td>
<td>0.2 ± 0.1</td>
<td>9.9 ± 2.1**</td>
</tr>
<tr>
<td>30 μM</td>
<td>75.5 ± 2.0</td>
<td>0.6 ± 0.3</td>
<td>23.9 ± 2.0**</td>
</tr>
<tr>
<td><strong>Rilpivirine</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0 μM</td>
<td>99.0 ± 0.2</td>
<td>0.1 ± 0.1</td>
<td>0.9 ± 0.2</td>
</tr>
<tr>
<td>3 μM</td>
<td>98.5 ± 0.2</td>
<td>0.1 ± 0.1</td>
<td>1.5 ± 0.2</td>
</tr>
<tr>
<td>10 μM</td>
<td>94.7 ± 1.1</td>
<td>0.1 ± 0.1</td>
<td>5.3 ± 1.1*</td>
</tr>
<tr>
<td>30 μM</td>
<td>73.2 ± 9.1</td>
<td>0.1 ± 0.1</td>
<td>26.7 ± 9.1***</td>
</tr>
<tr>
<td><strong>Efavirenz</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0 μM</td>
<td>99.4 ± 0.1</td>
<td>0.1 ± 0.1</td>
<td>0.4 ± 0.1</td>
</tr>
<tr>
<td>3 μM</td>
<td>98.9 ± 0.1</td>
<td>0.1 ± 0.1</td>
<td>0.9 ± 0.2</td>
</tr>
<tr>
<td>10 μM</td>
<td>97.2 ± 0.7</td>
<td>0.1 ± 0.1</td>
<td>2.7 ± 0.7*</td>
</tr>
<tr>
<td>30 μM</td>
<td>85.1 ± 2.1</td>
<td>4.0 ± 1.5*</td>
<td>10.9 ± 1.8***</td>
</tr>
</tbody>
</table>

Table 4.1  Quantification of apoptosis and necrosis by HPI staining following 24 hours treatment with increasing concentrations (3, 10 and 30 μM) of etravirine (A) rilpivirine (B) and efavirenz (C). Data was analysed with two-way ANOVAs and Bonferronis post-hoc test and is expressed as percentage of total cells counted ± SEM, n = 3-6. *p<0.05, **p<0.01 and ***p<0.001 etravirine, rilpivirine or efavirenz vs. untreated control.
4.3.2.2 Measurements of histones in the cytoplasm as a marker of apoptosis

Treatment of H9c2 cells with efavirenz for 24 hours followed by the cell death ELISA confirmed the results obtained following morphological analysis; 30 μM efavirenz led to an increase in histone in the cytoplasm indicative of apoptotic cell death (figure 4.2). The results also showed a statistically significant increase in histones following treatment with both 10 and 30 μM etravirine and 30 μM rilpivirine, which was not detected during morphological analysis. Treatment with 30 μM efavirenz did however lead to a significantly higher level of histones than treatment with both rilpivirine and etravirine (p<0.001), (figure 4.2).

![Graph](image)

**Figure 4.2** Cytoplasmic histone-associated-DNA-fragments as a marker of apoptotic cell death measured after 24 hours treatment with increasing concentrations (10 and 30 μM) of etravirine, rilpivirine and efavirenz. Data was analysed with two-way ANOVA and is expressed as mean percentage of control ± SEM, n = 3. *p<0.05, **p<0.01 and ***p<0.001 vs. untreated control, †††p<0.001 efavirenz vs. etravirine and rilpivirine.
4.3.2.3 Detection of caspase-3/7 activity

Caspase-3/7 activity increased significantly in H9c2 cells in response to treatment with 10 μM rilpivirine and etravirine as well as 30 μM rilpivirine, etravirine and efavirenz for 24 hours. Rilpivirine treatment led to a significantly higher increase of caspase-3/7 activation than both etravirine and efavirenz (figure 4.3).

![Caspase activity graph](image)

**Figure 4.3** Quantification of caspase-3/7 following 24 hours treatment with increasing concentrations (3, 10 and 30 μM) of etravirine, rilpivirine and efavirenz. All NNRTIs led to an increase in caspase-3/7 activity. Data was analysed with two-way ANOVA and Bonferroni's post hoc test and is expressed as mean percentage of control ± SEM, n = 3. *p<0.05 and ***p<0.001 etravirine, rilpivirine and efavirenz vs. untreated control, †††p<0.001 rilpivirine vs. etravirine and efavirenz.
4.3.3 Oxidative stress determination

4.3.3.1 Estimation of reactive oxygen species with the DCF assay

Oxidative stress, measured with DCF, decreased following treatment with rilpivirine and efavirenz in a dose dependent manner (table 4.2). Treatment with etravirine did not significantly change the level of oxidative stress in comparison to untreated cells. DCF was calculated according to the following formula: \( \frac{(F_{t28} - F_{t0}) \times 100}{(control~F_{t28} - F_{t0})} \) giving percentage change in fluorescence, where \( F_{t28} \) is fluorescence at 28 minutes and \( F_{t0} \) is fluorescence at 0 minutes.

<table>
<thead>
<tr>
<th></th>
<th>0 μM</th>
<th>3 μM</th>
<th>10 μM</th>
<th>30 μM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Etravirine</td>
<td>100 ± 3.4</td>
<td>91 ± 6.0</td>
<td>85 ± 7.6</td>
<td>92 ± 6.0</td>
</tr>
<tr>
<td>Rilpivirine</td>
<td>100 ± 2.1</td>
<td>87 ± 2.8</td>
<td>67 ± 3.8*</td>
<td>59 ± 1.3*</td>
</tr>
<tr>
<td>Efavirenz</td>
<td>100 ± 3.1</td>
<td>88 ± 8.9</td>
<td>83 ± 2.9</td>
<td>55 ± 5.5*</td>
</tr>
</tbody>
</table>

Table 4.2  Change in reactive oxygen species measured by DCF following 24 hours treatment with etravirine, rilpivirine and etravirine measured over a 28-minute period. Data was analysed with two-way ANOVA and is expressed as mean percentage of untreated cells ± SEM, n = 3. *p<0.05 rilpivirine and efavirenz vs. untreated control.

4.3.3.2 Estimation of reactive oxygen species with the NBT assay

The NBT assay was performed on H9c2 cells following treatment with efavirenz and rilpivirine. There was not an increase in oxidative stress following treatment with either drug (table 4.3).
### Table 4.3 Oxidative stress measured following treatment with different concentrations of efavirenz and rilpivirine (10 and 30 μM). Data was analysed with two-way ANOVA and is expressed as mean of percentage of untreated cells ± SEM, n=3, 3 replicates per experiment.

<table>
<thead>
<tr>
<th>Concentration (μM)</th>
<th>Rilpivirine</th>
<th>Efavirenz</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 μM</td>
<td>100±4.8</td>
<td>100 ± 3.2</td>
</tr>
<tr>
<td>10 μM</td>
<td>107 ± 2.6</td>
<td>96 ± 2.9</td>
</tr>
<tr>
<td>30 μM</td>
<td>106 ± 2.5</td>
<td>97 ± 3.2</td>
</tr>
</tbody>
</table>

#### 4.3.4 Measurement of CHOP, a marker of ER stress

Treatment of H9c2 cells with all three NNRTIs led to a dose dependent upregulation of ER stress marker protein CHOP in comparison to untreated cells. This was statistically significant following treatment with both 10 and 30 μM for efavirenz and for 30 μM for etravirine and rilpivirine. There was a significantly higher amount of CHOP in the protein extracted from the cells treated with 30 μM efavirenz in comparison to those treated with 30 μM rilpivirine and etravirine (figure 4.4).

The upregulation of CHOP was significant after 6 hours treatment with efavirenz, with levels peaking following 12 hours treatment and decreasing at 18 hours with a further decrease at 24 hours, see figure 4.5 below. A similar time frame for the upregulation of CHOP was seen following treatment with both etravirine and rilpivirine (figure 4.6 and 4.7).
Figure 4.4  Representative blot (A) of proteins extracted from H9c2 cells following treatment with increasing concentrations of etravirine, rilpivirine and efavirenz (3 – 30 µM) for 24 hours and densitometric analysis of the blot image (B). There was a dose dependent increase in the expression of the protein CHOP (top) in relation to β-actin (bottom) in response to all three NNRTIs, with efavirenz treatment leading to a significantly higher level of CHOP: β-actin than rilpivirine and etravirine. Data was analysed with two-way ANOVA and Bonferroni post hoc test, and is expressed as mean ± SEM, n=3. *p<0.05, **p<0.01 and ***p<0.001 etravirine, rilpivirine and efavirenz vs. untreated control.
Figure 4.5 Representative blot (A) of proteins extracted from H9c2 cells following treatment with increasing concentrations of efavirenz (3 – 30 µM) following 6, 12, 18 and 24 hours treatment and densitometric analysis of the blot image (B). Data was analysed with two-way ANOVA and Bonferroni post-hoc test and is expressed as mean ± SEM. n=3. ***p<0.001 efavirenz vs. untreated control, **p<0.01 efavirenz vs. untreated control and †p<0.05 efavirenz 12 hours vs. efavirenz 24 hours.
A.

<table>
<thead>
<tr>
<th></th>
<th>6h</th>
<th>12h</th>
<th>18h</th>
<th>24h</th>
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</tr>
<tr>
<td>30</td>
<td>3</td>
<td>10</td>
<td>30</td>
<td>3</td>
</tr>
</tbody>
</table>

CHOP: 31 kDa
β-actin: 38 kDa

B.

Figure 4.6 Representative blot (A) of proteins extracted from H9c2 cells following treatment with increasing concentrations of etravirine (3 – 30 µM) following 6, 12, 18 and 24 hours treatment and densitometric analysis of the blot image (B). Data was analysed with two-way ANOVA and Bonferroni post-hoc test and is expressed as mean ± SEM. n=3. *p<0.001 etravirine vs. untreated control, **p<0.01 etravirine vs. untreated control, ***p<0.001 etravirine vs. untreated control and †p<0.05 etravirine 12 hours vs. etravirine 24 hours.
A.

<table>
<thead>
<tr>
<th></th>
<th>6h</th>
<th>12h</th>
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<th>24h</th>
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</tr>
</tbody>
</table>

CHOP: 31kDa

β-actin: 38kDa

B.

Figure 4.7 Representative blot (A) of proteins extracted from H9c2 cells following treatment with increasing concentrations of rilpivirine (3 – 30 μM) following 6, 12, 18 and 24 hours treatment and densitometric analysis of the blot image (B). Data was analysed with two-way ANOVA and Bonferroni post-hoc test and is expressed as mean ± SEM. n=3. *p<0.05 rilpivirine vs. untreated control.
4.3.5 Effect of PARP inhibition on NNRTI induced loss of cell viability

Treatment with different doses of the PARP inhibitor PJ34 in combination with rilpivirine, efavirenz and etravirine did not improve cell viability in the MTT assay (table 4.4). Efavirenz was the only NNRTI that increased apoptotic cell death (table 4.5), which was partially reduced by 3 μM PJ34. Necrosis remained unchanged for all three drugs with the addition of PJ34 to the treatment media (table 4.5).
A.

<table>
<thead>
<tr>
<th>PJ34</th>
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</tr>
</thead>
<tbody>
<tr>
<td>0 μM</td>
<td>100 ± 2.3</td>
</tr>
<tr>
<td>0.3 μM</td>
<td>94 ± 1.2</td>
</tr>
<tr>
<td>1 μM</td>
<td>94 ± 2.3</td>
</tr>
<tr>
<td>3 μM</td>
<td>88 ± 1.5</td>
</tr>
</tbody>
</table>

B.

<table>
<thead>
<tr>
<th>PJ34</th>
<th>Rilpivirine</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 μM</td>
<td>100 ± 2.3</td>
</tr>
<tr>
<td>0.3 μM</td>
<td>94 ± 1.2</td>
</tr>
<tr>
<td>1 μM</td>
<td>94 ± 2.3</td>
</tr>
<tr>
<td>3 μM</td>
<td>88 ± 1.5</td>
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</table>

C.

<table>
<thead>
<tr>
<th>PJ34</th>
<th>Etravirine</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 μM</td>
<td>100 ± 2.3</td>
</tr>
<tr>
<td>0.3 μM</td>
<td>94 ± 1.2</td>
</tr>
<tr>
<td>1 μM</td>
<td>94 ± 2.3</td>
</tr>
<tr>
<td>3 μM</td>
<td>88 ± 1.5</td>
</tr>
</tbody>
</table>

Table 4.4  Cell viability results following simultaneous treatment with NNRTIs and PJ34 for 24 hours. Treatment with PJ34 in combination with efavirenz (A), rilpivirine (B) and etravirine (C) did not decrease loss of cell viability. Data are expressed as mean percentage of untreated cells ± SEM, analysed with two-way ANOVA, n = 2. *p<0.05 efavirenz, rilpivirine and etravirine vs. untreated control.
<table>
<thead>
<tr>
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<th>Alive</th>
<th>Apoptotic</th>
<th>Necrotic</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated</td>
<td>98.7 ± 0.5</td>
<td>0.1 ± 0.1</td>
<td>1.1 ± 0.5</td>
</tr>
<tr>
<td>30 μM rilpirine</td>
<td>90.4 ± 0.6</td>
<td>0.3 ± 0.1</td>
<td>9.2 ± 0.5*</td>
</tr>
<tr>
<td>30 μM rilpirine and 3 μM PJ34</td>
<td>90.5 ± 1.6</td>
<td>0.3 ± 0.1</td>
<td>9.3 ± 1.5</td>
</tr>
<tr>
<td>3 μM PJ34</td>
<td>98.1 ± 1.4</td>
<td>0.2 ± 0.2</td>
<td>1.7 ± 1.2</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>Alive</th>
<th>Apoptotic</th>
<th>Necrotic</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated</td>
<td>98.7 ± 0.5</td>
<td>0.1 ± 0.1</td>
<td>1.1 ± 0.5</td>
</tr>
<tr>
<td>30 μM etravirine</td>
<td>76.3 ± 1.6</td>
<td>0.2 ± 0.1</td>
<td>23.5 ± 1.6*</td>
</tr>
<tr>
<td>30 μM etravirine and 3 μM PJ34</td>
<td>77.3 ± 3.0</td>
<td>0.3 ± 0.1</td>
<td>22.4 ± 3.2</td>
</tr>
</tbody>
</table>

<table>
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<tr>
<th></th>
<th>Alive</th>
<th>Apoptotic</th>
<th>Necrotic</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated</td>
<td>98.7 ± 0.5</td>
<td>0.1 ± 0.1</td>
<td>1.1 ± 0.5</td>
</tr>
<tr>
<td>30 μM efavirenz</td>
<td>85.7 ± 3.2</td>
<td>6.9 ± 2.1*</td>
<td>7.4 ± 3.5*</td>
</tr>
<tr>
<td>30 μM efavirenz and 3 μM PJ34</td>
<td>89.5 ± 3.3</td>
<td>3.7 ± 1.8</td>
<td>6.8 ± 4.1</td>
</tr>
</tbody>
</table>

Table 4.5  Quantification of apoptosis and necrosis by HPI staining following 24 hours treatment with 30 μM rilpirine, etravirine and efavirenz with and without 3 μM PJ34. A decrease in apoptosis in efavirenz treated cells was seen in the presence of PJ34. Data was analysed with three separate two-way ANOVAs and Bonferroni post-hoc test and is expressed as percentage of total cells counted ± SEM, n = 3. *p<0.05  efavirenz, rilpirine and etravirine vs. untreated control.
4.3.6 Effect of PERK inhibitor, GSK 2606414, on NNRTI induced loss of cell viability

Addition of GSK 2606414 30 minutes prior to adding the NNRTIs for 24 hours did not protect against loss of cell viability induced by the NNRTIs (table 4.6).

<table>
<thead>
<tr>
<th></th>
<th>Untreated</th>
<th>30 µM etravirine</th>
<th>30 µM rilpivirine</th>
<th>30 µM efavirenz</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0 µM</td>
<td>0.1 µM</td>
<td>0.3 µM</td>
<td>1 µM</td>
</tr>
<tr>
<td>Untreated</td>
<td>100 ± 1.3</td>
<td>101 ± 0.8</td>
<td>99 ± 1.1</td>
<td>90 ± 1.0</td>
</tr>
<tr>
<td>30 µM etravirine</td>
<td>73 ± 0.9*</td>
<td>73 ± 0.8</td>
<td>71 ± 1.1</td>
<td>65 ± 1.5</td>
</tr>
<tr>
<td>30 µM rilpivirine</td>
<td>68 ± 1.7*</td>
<td>72 ± 1.4</td>
<td>74 ± 1.9</td>
<td>70 ± 2.1</td>
</tr>
<tr>
<td>30 µM efavirenz</td>
<td>45 ± 1.4*</td>
<td>47 ± 2.6</td>
<td>43 ± 2.2</td>
<td>44 ± 2.4</td>
</tr>
</tbody>
</table>

Table 4.6  Cell viability results following treatment with NNRTIs and GSK 2606414 for 24 hours. Treatment with the PERK inhibitor did not protect against loss of cell viability. Data was analysed with two-way ANOVA and is expressed as mean ± SEM, n = 3, 6 replicates per experiment. *p<0.05  etravirine, rilpivirine and efavirenz vs. untreated control.
4.4 Discussion

The results presented in this chapter confirm the results previously obtained in our lab with the first generation NNRTI efavirenz (Faltz 2013) on the myocardial H9c2 cell line. More importantly, similar to the endothelial effects discussed in chapter 3, the second generation NNRTIs etravirine and rilpivirine were also shown to have the same damaging effects as efavirenz, although to a lesser extent, on cell viability, apoptosis and ER stress. Interestingly necrosis levels were higher following treatment with the second generation NNRTIs than with efavirenz.

Treatment with all three NNRTIs led to a time and concentration dependent loss of cell viability in the MTT assay. In line with the results on EA.hy926 cells (see chapter 3), efavirenz led to a significantly higher loss of cell viability at 30 μM than etravirine and rilpivirine (52 % versus 35 % and 40 % respectively), whereas treatment with both etravirine and rilpivirine was more damaging than efavirenz following treatment with 10 μM (25 % and 20 % versus 10 % respectively). This indicates different underlying damaging mechanisms as discussed in chapter 3.

An increase in ROS could not be detected following treatment with efavirenz, etravirine or rilpivirine in the DCF assay or the NBT assay in the above experiments. Efavirenz has been shown to cause an increase in ROS in other cell lines and has been shown to inhibit complex I, as discussed in chapter 3.
In the above experiments, the DCF assay gave negative results, for both rilpivirine and efavirenz, whereas etravirine had little or no effect at the concentrations used. In theory, a long drug exposure time could lead to a loss of cell viability and therefore negative readings due to lack of cells in comparison to control wells. Another possible explanation lies in the chemical structure of DCF and the HIV drugs; as the decrease in oxidative stress decreases with increasing concentration of the drugs, there might be an interaction between the two, which prevents the assay from working. The NBT assay may not be sensitive enough, and methods isolating the mitochondria in order to measure the production of ROS would perhaps have been more appropriate.

Necrosis levels increased following treatment with all three NNRTIs. Interestingly necrosis levels were higher following treatment with both rilpivirine and etravirine in comparison to efavirenz (26.7 ± 9.1, 23.9 ± 2.2 and 10.9 ± 1.8 respectively, expressed as percentage of total cells counted). The PARP overactivation pathway which results in necrosis through ATP depletion was excluded in the above experiments, indicating that PARP activation through DNA damage may not be an underlying damaging mechanism of the NNRTIs. As already discussed, necrosis can also result in response to an increased production of reactive oxygen species, increased cytosolic calcium concentrations and activation of death receptors (Kung, Konstantinidis et al. 2011). An increase in ROS was not detected in the above experiments. Whereas ER stress is associated with disturbances to calcium
homeostasis, apoptosis rather than necrosis is normally associated with ER stress (Janssen, Horn et al. 2009), indicating that other molecular pathways beyond PARP overactivation, increased production of ROS and ER stress are involved.

The other mode of cell death considered in the above experiments, apoptosis, was increased following treatment with efavirenz (30 µM) but not etravirine or rilpivirine when analysed morphologically. However, treatment with all three NNRTIs (30 µM) led to a significant increase in histones in the media indicative of apoptosis, with efavirenz treatment increasing levels significantly more than both rilpivirine and etravirine (p<0.001). The ELISA that measures cytoplasmic-histone-associated-DNA-fragments may be a more sensitive assay for the detection for apoptosis, whereas morphological analysis following staining is a subjective method in which the investigator must identify the distinct appearance of apoptotic cells, which may account for the different results. Interestingly, it has been shown that there is a minimum of 12 hours time delay between apoptotic blebbing identified by staining with propidium iodide and detection of nucleosomes in culture media in Jurkat cells (Nieuwenhuijze, Lopik et al. 2003). This could indicate that the time frame for apoptosis is different between efavirenz in comparison to etravirine and rilpivirine, indicating a possible different underlying apoptotic mechanism/signalling pathway to apoptosis. Efavirenz has previously been shown to cause apoptosis in H9c2 cells in our lab (Faltz 2013), and the drug has also been shown to trigger the apoptotic cell death pathway in hepatocytes (Apostolova,
Apoptosis is linked to the development of a range of cardiovascular diseases (Kim and Kang 2010), and the results obtained in this study indicate that etravirine and rilpivirine may have a better cardiovascular profile than efavirenz.

Overactivation of PARP can lead to both necrosis and apoptosis. In fact, the PARP inhibitor PJ34 has been shown to decrease apoptosis triggered by efavirenz in H9c2 cells (Faltz 2013). There was a trend towards decreased apoptosis following treatment with efavirenz and PJ34 in the above experiment (measured morphologically) however statistical significance was not reached. It would have been interesting to use the combinations of NNRTIs and PJ34 on both the caspase-3/7 and cell death ELISA to determine if PJ34 would have had an effect on apoptosis levels in the cell death assay as well as effector caspase activation.

Another molecular pathway that can lead to apoptotic cell death is ER stress, which is often associated with the upregulation of CHOP (Oyadomari and Mori 2004). CHOP is also known as growth arrest- and DNA damage-inducible gene 153 (GADD153) and is a transcriptional factor and key component in ER stress, which is activated by all three UPR signalling pathways: the PERK pathway via ATF4, the IRE1α pathway via ASK1, JNK and MAPK1 P35 and through the ATF6 pathway directly via activated ATF6. In the above experiments, CHOP was significantly upregulated following treatment with 30 μM of all NNRTIs, with efavirenz leading to
a greater increase than etravirine and rilpivirine. Of the lower treatment concentrations, only 10 μM efavirenz led to a significant increase in CHOP. Stimulation of ER stress may explain why all three NRNTIS increased the levels of histones in the media indicative of apoptosis and furthermore why treatment with efavirenz lead to a greater degree of apoptosis than etravirine and rilpivirine. Efavirenz has previously been shown to cause ER stress in H9c2 cells in our lab and in hepatocytes (Apostolova, Gomez-Sucerquia et al. 2013), whereas the ER stress inducing effect of protease inhibitors have been known since 2005 and are well documented. The initial publication showed an increase in ER stress in mouse macrophages (Zhou, Pandak et al. 2005), followed by primary hepatocytes (Zhou, Gurley et al. 2006), human endothelial cells (Sun, Wu et al. 2009) and intestinal epithelial cells (Wu, Sun et al. 2010). More recently protease inhibitors have been shown to cause ER stress in peripheral blood mononuclear cells as well as a range of cell lines including liver, kidney, fibroblast and epithelial cell lines (Taura, Kariya et al. 2013).

CHOP expression was already seen following 6 hours treatment, peaked following 12 hours and decreased towards 24 hours for all NNRTIs. This indicates that the underlying mechanism leading to ER stress may be the same for all three drugs. In rat primary hepatocytes, CHOP peaked after 2 hours exposure to protease inhibitors (Zhou, Gurley et al. 2006) whereas in mouse macrophages, CHOP expression peaked following 6 hours treatment (Zhou, Pandak et al. 2005) and in embryonic kidney
cells ≥ 6 hours, as longer treatment periods were not performed (Taura, Kariya et al. 2013). These results indicate the response is cell specific. Ideally, timed mRNA CHOP levels could have been measured using rtPCR to confirm the data obtained from the Western Blots. In addition to using CHOP as a marker of ER stress, the results can be confirmed by measuring other members of the ER stress protein cascade, such as GRP78, ATF6 and IRE-1.

HIV proteins have also been shown to induce ER stress (Maingat, Halloran et al. 2011, Tiede, Cook et al. 2011). It may therefore be hypothesized that the NNRTIs potentiate these effects. Several studies suggest that ER stress signalling pathways are implicated in various cardiovascular diseases. Indeed, ER stress has been associated with cardiac hypertrophy and heart failure (Hamada, Suzuki et al. 2004, Okada, Minamino et al. 2004) as well as cardiac damage after ischaemia/reperfusion (Harpster, Bandyopadhyay et al. 2006), and is currently a topic gaining renewed research interest. A competitive inhibitor of PERK, GSK 2606414, which leads to a decrease in phosphorylated eIF1 and downstream CHOP, has been shown to be a potential promising novel agent in the treatment of tumours in mice-studies (Atkins, Liu et al. 2013). Animal experiments also show promising results using a PERK inhibitor in the treatment of ER stress associated with neurodegeneration (Moreno, Halliday et al. 2013). The PERK inhibitor used in the above experiments did however not protect against the loss of cell viability. However, as we did not determine whether the PERK inhibitor was able to protect against the increased
apoptosis observed with NNRTIs we are unable to comment on any possible protective effects inhibiting PERK may have and this should be investigated using morphological analysis and/or measurement of cytoplasmic histones. As it is likely that NNRTI-mediated impairment of mitochondrial function occurs before ER stress this may explain why no protective effect on the cell viability assay was observed.

In conclusion, treatment with all NNRTIs led to ER stress, conceivably subsequent to mitochondrial damage, and was followed by apoptosis through a caspase dependent pathway. An increase in ROS and overactivation of PARP did not appear to be mechanisms involved in the damaging effects of the NNRTIs. These results show that the second generation NNRTIs etravirine and rilpivirine have the same effects on cardiac cells as the first generation efavirenz, although to a lesser extent, suggesting that they may have a safer cardiovascular side effect profile.
Chapter Five: The effects of abacavir on the rat myocardial cell line H9c2
5.1 Introduction

In 2008, The Data Collection on Adverse Events of Anti-HIV Drugs (D:A:D) study group initiated a debate with a publication that identified an association between abacavir use and an increased risk of myocardial infarction (Group, Sabin et al. 2008). Several studies have since supported the findings of the D:A:D study. These include the SMART study (Strategies for Management of Anti-Retroviral Therapy and Groups 2008), the Simplification with Tenofovir-Emtricitabine or Abacavir-Lamivudine Study, STEAL, (Martin, Bloch et al. 2009) the analysis of The French Hospital Database on HIV (Lang, Mary-Krause et al. 2010) and the analysis of databases from Quebec's Public Health Insurance Database (QPHID) (Durand, Sheehy et al. 2011). However, in contrast to the above studies, The AIDS Clinical Trial Group (Ribaudo, Benson et al. 2011), the Veterans Administration's Clinical Case Registry (Bedimo, Westfall et al. 2011) and Glaxo-Smith-Klein (Brothers, Hernandez et al. 2009) did not find an association between the use of abacavir and myocardial infarction.

Following the D:A:D study other studies have tried to identify a link between abacavir use and increased risk factors for myocardial infarction thereby explaining the association, however the results have been ambiguous. For example studies on lipids have shown that treatment with abacavir lead to increases in TG compared to untreated HIV positive women as well as HIV negative women (Anastos, Lu et al. 2007). RAVE, BICOMBO and several other studies have shown that abacavir has a
less favourable effect on the lipid profile with increases in LDL, TG and TC than tenofovir whereas a positive increase in HDL was observed (Martinez, Arranz et al. 2009, Rasmussen, Tolstrup et al. 2011, Saumoy, Ordonez-Llanos et al. 2011, Pammi, Arumainayagam et al. 2013). On the other hand abacavir has been shown to improve the lipid profile when replacing a protease inhibitor, by decreasing TG, LDLc and TC, with no effects on HDLc (Keiser, Sension et al. 2005).

Analyses of biomarkers associated with activation of different pathways implicated in CVD pathogenesis have also been inconclusive, with some studies reporting an increase and other studies reporting a decrease of the same biomarkers following abacavir exposure (see appendix 1).

Furthermore, a definite underlying pathological molecular mechanism has not yet been established for abacavir mediated myocardial damage. To date, the published studies have mostly focused on the indirect effects of abacavir, for example effects on lipid profile, in the development of cardiovascular pathology. The aim of this chapter is therefore to investigate the direct effects of abacavir on the myocardium, as a proposed mechanism underlying the increased risk of myocardial infarctions in this patient group.
5.2 Experimental protocols

H9c2 cells were treated with different concentrations of abacavir (3, 10, 30 or 100 μM), palmitic acid (10, 30, 100, 300, 500 or 1000 μM), cobalt chloride (50, 100, 200, 400, 600 or 800 μM) or a combination of abacavir with either palmitic acid or cobalt chloride.

Palmitic acid was prepared as a conjugate with bovine serum albumin prior to dissolution in media, with or without abacavir. 27 mg palmitic acid was added to 5 ml dH2O, 200 μL 70 % ethanol, and 200 μL 0.1 N NaOH and boiled at 100 °C until the palmitic acid had dissolved. 1 ml of the resultant solution was mixed with 1 ml 6 mM albumin to give a 10 mM palmitic acid solution with 3 mM albumin. Different concentrations of cobalt chloride were added to the treatment media, alongside abacavir.

The MTT assay as described in methods section 2.3.3 was employed to measure cell viability. In addition a second set of experiments were done, in which the H9c2 cells were pretreated with abacavir for 24 hours prior to adding the combination of abacavir and palmitic acid/cobalt chloride for 24 hours. HPI staining was used to evaluate mode of cell death in 24 well plates (40 000 cells/well) and Western blotting carried out to determine the presence of ER stress marker CHOP as explained in method section 2.3.4 and 2.3.12 respectively.
5.3 Results

5.3.1 Effect of abacavir on H9c2 cells

Following 24 hour treatment with abacavir, the highest concentration (100 μM) was the only dose that led to a significant loss of cell viability on the MTT assay, whereas both 30 and 100 μM resulted in a significant loss after a 48 hour treatment period (figure 5.1).

HPI staining showed no increase in apoptosis or necrosis following exposure to increasing concentrations of abacavir (table 5.1).

Western blot performed on proteins extracted following 24 hour treatment with increasing concentrations of abacavir (3, 10 and 30 μM), showed that abacavir did not increase CHOP, a marker of ER stress (figure 5.2).
Figure 5.1  Effect of abacavir on H9c2 cell viability following treatment with increasing concentrations of abacavir for 24 hours and 48 hours. Graph shows a statistically significant time and dose dependent reduction in cell viability. Data is expressed as mean percentage of untreated cells ± SEM, and was analysed with two-way ANOVA and Bonferroni post-hoc test, n = 3-4, 6 replicates per experiment, *p<0.05 abacavir vs. untreated cells, † p<0.05 24 hours vs. 48 hours treatment with abacavir, ^ p<0.05 abacavir 30 µM vs. abacavir 100 µM.
Table 5.1  Quantification of apoptosis and necrosis following 24 hours treatment with increasing concentrations (3, 10 and 30 µM) of abacavir. There was not a statistically significant increase in apoptosis or necrosis at the concentrations used in comparison to untreated cells. Data is expressed as percentage of total cells counted ± SEM and analysed with two-way ANOVA, n = 3.

<table>
<thead>
<tr>
<th>Abacavir</th>
<th>Alive</th>
<th>Apoptotic</th>
<th>Necrotic</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 µM</td>
<td>99.2 ± 0.3</td>
<td>0.1 ±0.1</td>
<td>0.7 ± 0.4</td>
</tr>
<tr>
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<td>1.0 ± 0.2</td>
</tr>
<tr>
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<td>0.0 ± 0.0</td>
<td>0.3 ± 0.1</td>
</tr>
<tr>
<td>30 µM</td>
<td>99.2 ± 0.2</td>
<td>0.1 ± 0.1</td>
<td>0.7 ± 0.2</td>
</tr>
</tbody>
</table>
A. Abacavir [μM]

<table>
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<th>Abacavir [μM]</th>
<th>CHOP</th>
<th>β-actin</th>
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</thead>
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</tr>
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<td><img src="38kDa" alt="β-actin Image" /></td>
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</tr>
<tr>
<td>30</td>
<td><img src="31kDa" alt="CHOP Image" /></td>
<td><img src="38kDa" alt="β-actin Image" /></td>
</tr>
</tbody>
</table>

B.

![Bar Chart Image]

Figure 5.2 Representative blot (A) of proteins extracted from H9c2 cells following treatment with increasing concentrations of abacavir (3 – 30 μM) for 24 hours and densitometric analysis of the blot image (B). Expression of the protein CHOP (top) in relation to β-actin (bottom) was not significantly different between cells treated with abacavir and untreated cells. Data was analysed with one-way ANOVA, n = 2.
5.3.2 Effect of cobalt chloride on H9c2 cells

There was a dose dependent drop in cell viability after 24 hours treatment with 200 μM cobalt chloride. The lower doses did not alter viability in a statistically significant manner (figure 5.3).

Apoptosis and necrosis remained unchanged following treatment with up to 400 μM, shown by HPI staining (table 5.2).

There was an increase in ER stress marker protein CHOP following 24 hours treatment. This was statistically significant with both 400 and 600 μM cobalt chloride (figure 5.4).
Figure 5.3  Effect of increasing concentrations of cobalt chloride on H9c2 cell viability. Graph shows a statistically significant dose dependent reduction in cell viability. Data is expressed as mean percentage of control ± SEM and was analysed with one-way ANOVA and Tukeys post-hoc test, n = 2-5, 6 replicates per experiment, *p<0.05 cobalt chloride vs. untreated cells.
Table 5.2  Quantification of apoptosis and necrosis following 24 hours treatment with increasing concentrations of cobalt chloride. There was not a statistically significant increase in apoptosis or necrosis at the concentrations used in comparison to untreated cells. Data is expressed as percentage of total cells counted ± SEM and was analysed with two-way ANOVAs, n = 2-4.
Figure 5.4 Representative blot (A) of proteins extracted from H9c2 cells following treatment with increasing concentrations of cobalt chloride for 24 hours and densitometric analysis of the blot image (B). There was an increase in ER stress protein marker CHOP in response to increasing concentrations of cobalt chloride, expressed as CHOP: β-actin. Data is expressed as mean ± SEM and was analysed with one-way ANOVA, n = 3, *p<0.05 and ***p<0.001 treated cells vs. untreated control, ††† p<0.001 cells treated with 400 μM versus cells treated with 600 μM.
5.3.3 Effect of abacavir in combination with cobalt chloride on H9c2 cells

There was no increase in damage following simultaneous exposure of H9c2 cells to a combination of abacavir and cobalt chloride in comparison to exposure to cobalt chloride/abacavir alone, on cell viability (table 5.3), apoptosis and necrosis (table 5.4) and expression of ER stress marker protein CHOP (figure 5.5).

Furthermore pretreatment of H9c2 cells with abacavir for 24 hours prior to treatment with the combination of abacavir and cobalt chloride, did not significantly enhance loss of cell viability (table 5.5).
<table>
<thead>
<tr>
<th>Cobalt chloride</th>
<th>0 µM</th>
<th>3 µM</th>
<th>10 µM</th>
<th>30 µM</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 µM</td>
<td>100 ± 1.7</td>
<td>97.8 ± 1.7</td>
<td>98.0 ± 2.5</td>
<td>92.1 ± 1.3</td>
</tr>
<tr>
<td>50 µM</td>
<td>95.9 ± 1.5</td>
<td>96.9 ± 1.7</td>
<td>93.6 ± 1.5</td>
<td>90.1 ± 1.4</td>
</tr>
<tr>
<td>100 µM</td>
<td>91.3 ± 1.5</td>
<td>94.6 ± 1.3</td>
<td>88.5 ± 1.3</td>
<td>90.4 ± 1.4</td>
</tr>
<tr>
<td>200 µM</td>
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<td>87.6 ± 1.1</td>
<td>81.5 ± 1.5</td>
<td>83.0 ± 1.3</td>
</tr>
</tbody>
</table>

Table 5.3  Cell viability results following treatment with a combination of increasing concentrations of abacavir (3, 10 and 30 µM) in combination with cobalt chloride (50, 100 and 200 µM). There was not an enhanced loss of cell viability with the combination of abacavir and cobalt chloride. Results are expressed as mean ± SEM. Results were analysed with two-way ANOVA and Bonferroni post-hoc test, n = 2, 6 replicates per experiment, *p<0.05 cobalt chloride vs. untreated cells.
### Table 5.4

Apoptosis and necrosis results following treatment with abacavir in combination with cobalt chloride (CoCl₂): A. Live cells B. Apoptotic cells C. Necrotic cells. There was not a statistically significant increase in apoptosis or necrosis. Results are expressed as mean ± SEM and analysed with three two-way ANOVAs, n=2.

<table>
<thead>
<tr>
<th></th>
<th>Abacavir</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
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<td></td>
<td>0 µM</td>
<td>10 µM</td>
<td>30 µM</td>
</tr>
<tr>
<td><strong>Cobalt chloride</strong></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>0 µM</td>
<td>99.9 ± 0.1</td>
<td>99.7 ± 0.1</td>
<td>99.2 ± 0.2</td>
</tr>
<tr>
<td>200 µM</td>
<td>99.3 ± 0.2</td>
<td>99.5 ± 0.3</td>
<td>99.9 ± 0.1</td>
</tr>
<tr>
<td>400 µM</td>
<td>99.2 ± 0.4</td>
<td>98.7 ± 0.5</td>
<td>98.9 ± 0.1</td>
</tr>
<tr>
<td>0 µM</td>
<td>0.0 ± 0.0</td>
<td>0.0 ± 0.0</td>
<td>0.1 ± 0.1</td>
</tr>
<tr>
<td>200 µM</td>
<td>0.3 ± 0.1</td>
<td>0.0 ± 0.0</td>
<td>0.0 ± 0.0</td>
</tr>
<tr>
<td>400 µM</td>
<td>0.2 ± 0.2</td>
<td>0.2 ± 0.2</td>
<td>0.3 ± 0.1</td>
</tr>
<tr>
<td>0 µM</td>
<td>0.1 ± 0.1</td>
<td>0.3 ± 0.1</td>
<td>0.7 ± 0.2</td>
</tr>
<tr>
<td>200 µM</td>
<td>0.4 ± 0.1</td>
<td>0.5 ± 0.3</td>
<td>0.1 ± 0.1</td>
</tr>
<tr>
<td>400 µM</td>
<td>0.6 ± 0.3</td>
<td>1.2 ± 0.3</td>
<td>0.8 ± 0.1</td>
</tr>
</tbody>
</table>
Figure 5.5 Representative blot (A) of proteins extracted from H9c2 cells following treatment with increasing concentrations of abacavir and cobalt chloride for 24 hours and densitometric analysis of the blot image (B). Expression of the protein CHOP (top) in relation to β-actin (bottom) was not significantly different between cells treated with cobalt chloride and cells treated with abacavir in combination with cobalt chloride. Data was analysed with two-way ANOVA, n = 2.
Table 5.5  Cell viability results following 24 hours pretreatment with abacavir (3, 10 and 30 μM) prior to treatment with the combination of cobalt chloride and abacavir. There was not an increase in loss of cell viability following the pretreatment period regimes in comparison to treating with either drug alone. Analysed with two-way ANOVA and Bonferroni post-hoc test. Data is expressed as mean percentage of control ± SEM, n = 3, 6 replicates per experiment, *p<0.05 cobalt chloride/abacavir vs. untreated cells.

<table>
<thead>
<tr>
<th>Cobalt chloride</th>
<th>0 μM</th>
<th>3 μM</th>
<th>10 μM</th>
<th>30 μM</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 μM</td>
<td>100 ± 0.7</td>
<td>96.0 ± 0.9</td>
<td>93.3 ± 0.9</td>
<td>85.5 ± 0.8*</td>
</tr>
<tr>
<td>50 μM</td>
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<td>89.4 ± 0.85</td>
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<td>74.3 ± 4.6</td>
<td>72.0 ± 3.8</td>
<td>71.4 ± 3.5</td>
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</table>
5.3.4 Effect of palmitic acid on H9c2 cells

There was a dose-dependent decrease in cell viability following treatment with palmitic acid. Treatment with 1000 μM (not shown on graph) decreased cell viability further, to 32 % ± 1.9 (figure 5.5). 100 μM palmitic acid caused a significant increase in cell death by necrosis and there was a trend towards significance for apoptosis, whereas treatment with 10 and 30 μM had no effect (table 5.6). There was also a dose dependent increase in ER stress protein CHOP (figure 5.6).
Figure 5.6  Effect of increasing concentrations (10 – 500 µM) of palmitic acid on H9c2 cell viability after 24 hours treatment. The graph shows a statistically significant dose dependent reduction in cell viability. Data is expressed as mean percentage of control ± SEM, n = 3, 6 replicates per experiment, analysed with one-way ANOVA and Tukeys post-hoc test. *p<0.05 Palmitic acid vs. untreated control.
Table 5.6  Quantification of apoptosis and necrosis following 24 hours treatment with increasing concentrations of palmitic acid. There was a statistically significant increase in necrosis in cells treated with 100 μM palmitic acid in comparison to untreated cells. Data is expressed as percentage of total cells counted ± SEM, n =3, and analysed with two-way ANOVA and Bonferroni post-hoc test, ***p<0.001 Palmitic acid vs. untreated control.

<table>
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<th>Palmitic acid (μM)</th>
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<tr>
<td>100</td>
<td>89.1 ± 1.2</td>
<td>1.9 ± 1.0</td>
<td>9.0 ± 1.5***</td>
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A. Palmitic acid [μM]

<table>
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<tbody>
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<td>β-actin</td>
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</tr>
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</table>

B.

Figure 5.7 Representative blot of proteins extracted from H9c2 cells following treatment with increasing concentrations of palmitic acid (50 to 500 μM). A. Western blot image and B. Densitometric analysis of the western blot image. There was a dose dependent increase in ER stress protein marker CHOP in response to palmitic acid, expressed as CHOP: β-Actin. Data is expressed as mean ± SEM and was analysed with one-way ANOVA, n = 2, *p<0.05 and ***p<0.001 treated cells vs. untreated control, ††† p<0.001 cells treated with 300 μM versus cells treated with 500 μM.
5.3.5 Effect of abacavir in combination with palmitic acid on H9c2 cells

There was no increase in damage following simultaneous exposure of H9c2 cells to a combination of abacavir and palmitic acid in comparison to exposure to palmitic acid/abacavir alone, on cell viability (table 5.7), apoptosis and necrosis (table 5.8) and expression of ER stress marker protein CHOP (figure 5.8).

Lastly, pretreatment of the H9c2 cells with different concentrations of abacavir for 24 hours prior to treatment with palmitic acid in combination with abacavir for 24 hours did not significantly enhance loss of cell viability (table 5.9).

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<tr>
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Table 5.7 Cell viability results following simultaneous treatment with palmitic acid and abacavir for 24 hours. Treatment of H9c2 cells with both abacavir and palmitic acid did not lead to an enhanced loss of cell viability. Data is expressed as mean percentage of control ± SEM, n = 2, 6 replicates per experiment, and was analysed with two-way ANOVA and Bonferroni post-hoc test, *p<0.05 palmitic acid vs. untreated cells.
### Table 5.8 Apoptosis and necrosis results following treatment with abacavir in combination with palmitic acid (PA): A. Live cells B. Apoptotic cells C. Necrotic cells.

There was not a statistically significant increase in necrosis between the cells treated with palmitic acid and the cells treated with a combination of palmitic acid and abacavir. Apoptosis remained unchanged. Results are expressed as mean ± SEM and were analysed with three one-way ANOVAs, and Tukeys post-hoc test, n=2. *p<0.05 palmitic acid vs. untreated cells

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</tr>
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<tr>
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<td>86.6 ± 1.8</td>
<td>89.4 ± 3.2</td>
<td>85.1 ± 0.6*</td>
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<td>B.</td>
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</tr>
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<td></td>
<td></td>
</tr>
<tr>
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<td>0.7 ± 0.2</td>
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<tr>
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</tbody>
</table>
Figure 5.8  Representative blot of proteins extracted from H9c2 cells following 24 hours treatment with a combination of abacavir (10 and 30 μM) and palmitic acid (30 and 100 μM). Expression of the protein CHOP (top) in relation to β-actin (bottom) was not significantly different between cells treated with abacavir or palmitic acid and the combination of abacavir and palmitic acid, n=2.
<table>
<thead>
<tr>
<th>Palmitic acid</th>
<th>Abacavir</th>
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<tbody>
<tr>
<td></td>
<td>0 µM</td>
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<tr>
<td>0 µM</td>
<td>100 ± 0.7</td>
</tr>
<tr>
<td>10 µM</td>
<td>99.5 ± 1.3</td>
</tr>
<tr>
<td>30 µM</td>
<td>102.3 ± 1.0</td>
</tr>
<tr>
<td>100 µM</td>
<td>87.8 ± 3.5*</td>
</tr>
</tbody>
</table>

Table 5.9  Cell viability results following 24 hours pretreatment with abacavir (3, 10 and 30 µM) prior to treatment with the combination of abacavir (3, 10 and 30 µM) and palmitic acid (10, 30 and 100 µM) for 24 hours. Pretreatment with abacavir did not enhance the damaging effects of palmitic acid on cell viability. Data was analysed with two-way ANOVA and Bonferroni post-hoc test. Data is expressed as mean ± SEM, n = 2, 6 replicates per experiment. *p<0.05 palmitic acid vs. untreated cells.
5.4 Discussion

The data presented in this chapter show that abacavir treatment did not have a direct damaging effect on cardiac at physiological concentrations. Furthermore, abacavir did not increase susceptibility of the cardiac myocytes to the damaging effects of hypoxia, induced chemically by cobalt chloride, or to the saturated fatty acid palmitic acid in the above experiments.

Abacavir is a carbocyclic 2’-deoxyguanosine nucleoside reverse transcriptase inhibitor, which is converted intracellularly to the active carbovir-triphosphate. About 50% of the drug binds to plasma proteins (Yuen, Weller et al. 2008). The D:A:D study showed the risk of myocardial infarction increased twofold soon after starting abacavir treatment and decreased after cessation of therapy indicating an acute effect, which is reversible and not cumulative (Group, Sabin et al. 2008). The risk was still significant after adjustments for risk to coronary heart disease implying that the mechanism is not due to abacavir altering lipid levels. This is supported by the fact that an increased risk of thrombotic stroke has not been identified.

In the above experiments abacavir did not have a direct damaging effect on rat cardiac cells. Abacavir did not increase apoptosis or necrosis, nor increase ER stress, even at the highest concentrations (30 μM and 100 μM respectively). Furthermore, the $C_{\text{max}}$ for abacavir is 6.9μM (DiCenzo, Forrest et al. 2003) and cell viability was
not decreased at clinical concentrations following 24 hours exposure. A reduction in
cell viability was seen following treatment with 100 μM, a concentration more than
10x the C_max. The MTT assay results can be interpreted as a marker of possible
mitochondrial dysfunction (Brand and Nicholls 2011), the above results therefore
indicates that abacavir does not affect mitochondrial function. Indeed, an abstract
presented at the British Pharmacological Society conference confirm this result. It
showed that cell viability measured with Hoescht staining did not change following
treatment with abacavir. Furthermore, the results in the study also concluded that
abacavir did not increase free radical generation, nor alter intracellular calcium
concentrations or mitochondrial membrane potential (Spiers 2008). A study in mice
has also indicated a lack of mitochondrial damage following abacavir exposure by
showing that myocardial mtDNA synthesis and levels of mtDNA are not altered by
abacavir treatment in cardiac tissue (Kohler, Hosseini et al. 2010). Abacavir has also
been shown not to cause mitochondrial damage in hepatocytes (Venhoff, Setzer et
al. 2007), human skeletal muscle (Saitoh, Haas et al. 2008), Human skin fibroblasts
and murine preadipocytes (Caron, Auclairt et al. 2008). On the other hand, HIV
drugs belonging to the same class, namely stavudine and zidovudine, have been
shown to cause mitochondrial dysfunction. They inhibit DNA polymerase-γ, the
enzyme responsible for synthesising proteins involved in mitochondrial electron
transport from the mitochondrial genome. This results in a decrease in
mitochondrial proteins, which form part of the electron transport complex and an
increase in reactive oxygen species (Day and Lewis 2004). A study showed that
mtDNA in blood mononuclear cells increased when changing therapy from other NRTIs to abacavir, supporting the above studies (Curran, Martinez et al. 2012).

If abacavir exposure doesn’t damage cardiac cells directly, does it perhaps predispose cardiac myocytes to increased damage by other stimuli, such as fatty acids? Palmitic acid is one the most abundant unsaturated fatty acid in a Western diet. Interestingly, The World Health Organisation has identified palmitic acid as a fatty acid associated with an increased risk of cardiovascular diseases (WHO 2003). A more recent review concluded modification of the dietary fat consumption away from saturated fat reduces the risk of cardiovascular disease (Hooper, Summerbell et al. 2012). Furthermore a large prospective study recently showed that a gradual increase in TG levels corresponded with a gradual increase in myocardial infarctions (Langsted, Freiberg et al. 2011). Specifically for the HIV population, Worm et al have shown that an increase in TG levels is a small independent risk factor for myocardial infarctions (Worm, Kamara et al. 2011). Interestingly, the HIV population has higher TG levels than the uninfected population (Souza, Luzia et al. 2013). Furthermore, abacavir exposure consistently increases TG levels further (Anastos, Lu et al. 2007). Although saturated fatty acids are the primary energy source for myocytes (Oliver 2006), excess is lipotoxic.

Previous work has demonstrated that palmitic acid reduces cell viability in the MTT assay (Cetrullo, Tantini et al. 2012) and leads to apoptosis in H9c2 cells (de Vries,
Vork et al. 1997, Cetrullo, Tantini et al. 2012). The above experiments confirm these results: A dose dependent loss of cell viability was seen following treatment with palmitic acid, and an increase approaching significance was seen in apoptosis following cell staining with propidium iodide/Hoescht. ER stress in H9c2 cells following treatment with palmitic acid was first reported in 2006 (Borradaile, Buhman et al. 2006), and this result was duplicated in the current study where a dose dependent increase in ER stress marker protein CHOP was observed in response to palmitic acid. ER stress activates the unfolded protein response, which is seen in mouse hearts and neonatal rat ventricular cells during induced myocardial infarction (Thuerauf, Marcinko et al. 2006).

It was proposed that abacavir might increase the risk of myocardial infarction by increasing the susceptibility to cardiac myocyte fatty acid damage. Palmitic acid has been shown to increase the production of reactive oxygen species, with subsequent ER stress and cell death in rat cardiac myocytes (Borradaile, Buhman et al. 2006). However, in the above experiments, abacavir did not predispose the cardiac myocytes to further palmitic acid mediated damage in cell viability, apoptosis and necrosis levels and ER stress. Indeed pretreatment with abacavir did not reduce cell viability over and above palmitic acid alone, indicating that abacavir use does not make the myocytes more prone to damage by palmitic acid.
The second hypothesis investigated if abacavir exposure would make the myocytes more prone to ischaemia, thereby worsening coronary artery disease. During myocardial infarctions, hypoxia lead to activation of hypoxia-inducible factor-1 (HIF-1α), a transcriptional factor which in non-hypoxic states is rapidly degraded, but in hypoxic states is stabilised and leads to transcriptional induction of genes involved in angiogenesis and cell survival (Ke and Costa 2006). Cobalt chloride is a chemical hypoxia-inducing agent, which mimics hypoxia by preventing degradation of HIF-1α (Epstein, Gleadle et al. 2001). Cobalt chloride has previously been used to mimic hypoxic conditions in H9c2 cells (Tong, Wu et al. 2012), and was used in the above experiments to determine if abacavir would enhance hypoxia in the myocytes.

Cobalt chloride treatment decreased cell viability in a dose dependent manner however no increase in apoptosis and necrosis was seen at the concentrations used (up to 400 μM). This is interesting, as ER stress following treatment with 400 μM cobalt chloride is seen in the above experiments. ER stress has been linked to apoptosis, therefore possible higher concentrations, longer exposure or a more sensitive method would lead to the detection of apoptosis. Indeed treatment with CoCl₂ has shown to lead to a dose dependent increase in apoptosis in H9c2 cells following treatment with 600 μM to 1200 μM CoCl₂ (Shu, Yang et al. 2008).
Ischaemia has been shown to lead to ER stress and activation of the unfolded protein response with an increase in CHOP levels in mouse hearts and H9c2 cells (Thuerauf, Marcinko et al. 2006). Interestingly the ER stress inducers thapsigargin, tunicamycin and brefeldin increase the levels of HIF-1α mRNA (Werno, Zhou et al. 2008).

The combination of abacavir with cobalt chloride was used to determine if abacavir exposure increased the risk of cardiac myocyte damage in response to hypoxia. The above results show that there was no enhancement in loss of cell viability following both simultaneous treatment and pretreatment, ER stress, apoptosis and necrosis over and above treating H9c2 cells with cobalt chloride alone. This indicates that the effects of hypoxia are not exacerbated by abacavir exposure.

In conclusion the above experiments show that the possible abacavir mediated increase in myocardial infarction, is not caused by abacavir directly affecting the myocytes in terms of increasing apoptosis and necrosis, increasing ER stress or making the cells more prone to the damaging effects of palmitic acid and hypoxia. A study in mice has concluded that abacavir has no effect on left ventricle mass and left ventricle end diastolic dimension (Kohler, Hosseini et al. 2010), however no studies have to date looked at electrical currents, and the possibility of abacavir altering these and thereby leading to myocardial damage.
Several other possible biological mechanisms linking cardiovascular diseases with abacavir have been suggested. The platelet hyper-reactivity theory was first reported by Satchell et al (Satchell, O’Halloran et al. 2011) and was followed by a study, which showed the active abacavir metabolite carbovir inhibited the enzyme soluble guanylyl cyclase. This in turn leads to platelet hyper-reactivity (Baum, Sullam et al. 2011). In support of the theory, a recent study in humans has shown increases in platelet activation markers in abacavir treated patients (Falcinelli, Francisci et al. 2013). This is a plausible mechanism as the proposed risk of myocardial infarction following abacavir exposure is not cumulative and reversible upon cessation of the NRTI. Furthermore it is patients that have already been categorized as having a high risk of cardiovascular diseases that seem to suffer from an increased risk of myocardial infarction following abacavir exposure. The platelet hyper-reactivity theory suggests abacavir leads to an increased risk of plaque rupture in this patient group.

Another suggested mechanism is related to the hyper-reactivity reaction seen in patients treated with abacavir. This is a serious reaction occurring in 5 % of patients treated with abacavir (Clay, 2002) necessitating cessation of abacavir therapy. In 90 % of this subset of abacavir treated patients, the hypersensitivity reaction is detected within the first 6 weeks of treatment, whereas in the remaining 10 % it is believed that it leads to a chronic sub clinical inflammatory response, which could
contribute to the increased risk of cardiovascular diseases (Mallal, Phillips et al. 2008).

A third hypothesis is the toxic abacavir metabolite explanation, which proposes that the increase in cardiovascular diseases is due to a highly reactive abacavir aldehyde metabolite, which is cardiotoxic (Charneira, Godinho et al. 2011, Charneira, Grilo et al. 2012, Grilo, Antunes et al. 2013).

The effects of abacavir on endothelial function are conflicting. Ex vivo animal and in vitro studies have shown that abacavir exposure causes an increase in superoxide levels, a decrease in eNOS levels and endothelial dysfunction in porcine pulmonary arteries and in human pulmonary arterial endothelial cells (Wang, Chai et al. 2009). Supporting the animal and in vitro cell studies is a human study on flow mediated vasodilation, a measure of endothelial function, in which HIV patients receiving abacavir versus those that were on a regime exclusive of abacavir, had significantly reduced flow mediated dilation (Hsue, Hunt et al. 2009). Flow mediated dilation is a measure of endothelium derived NO, which in turn inhibits platelet aggregation and adhesion. A decrease in NO could therefore lead to an increase in platelet activation, supporting the above platelet hyperactivity theory. Furthermore, in vitro experiments have shown an increase in leukocyte accumulation and interaction with endothelial cells following abacavir exposure (De Pablo, Orden et al. 2010), an indicator of endothelial dysfunction and an early event in the formation of
atherosclerosis. Interestingly this is also seen with didanosine, another NRTI associated with an increased risk of myocardial infarction in the D:A:D study, but not with the NRTIs tenofovir, emtricitabine or lamivudine, which have not been associated with an increased risk of myocardial infarction (De Pablo, Orden et al. 2010, De Pablo, Orden et al. 2012). Contradicting the above studies is a study on human coronary artery cells by Kim et al which found no evidence of an increase in pro-inflammatory markers, reactive oxygen species or markers of apoptosis following abacavir exposure (Kim, Gupta et al. 2011).

Following the D:A:D study and other supportive publications of abacavir identifying a possible epidemiological association with myocardial infarction, the place of abacavir in treatment of HIV changed from a first line NRTI to a second line, with a significant drop in the number of patients initiated on abacavir as a part of their treatment regime (Antoniou, Gillis et al. 2013). It has been shown here that abacavir doesn't have any direct effects nor does it predispose the cardiac myocytes to other toxic insults such as ischaemia and fatty acids. The underlying molecular mechanism has yet to be elucidated.
Chapter Six: General Discussion
6.1 Introduction

On a global scale, the HIV pandemic disproportionality affects low and middle-income countries, with over 24.7 million infected individuals living in Sub-Saharan Africa (WHO statistics from 2013). In 2013, of the 13 million people receiving antiretroviral therapy, 12 million were from low and middle-income countries (figure 6.1). New HIV infection rates are exceeding death rates, which together with initiatives to ensure antiretroviral therapy becomes more readily available means the number of people receiving antiretroviral therapy is expected to increase. Improving affordability therefore becomes a big driver. In a broader sense, improving affordability includes using treatment with the lowest possible drug toxicity. This warrants investigating and comparing the different effects the NNRTIs and abacavir have on the cardiovascular system to determine their cardiovascular profiles, in vitro and in vivo.
6.2 Main findings

- Abacavir did not lead to a loss of cell viability, apoptosis or necrosis or cause ER stress, neither did abacavir enhance the damaging effects of a diet high in fatty acids (mimicked by palmitic acid) or hypoxia (mimicked by cobalt chloride) on the H9c2 cells in vitro.

- This is the first time cobalt chloride as a model system for hypoxia has been shown to cause ER stress in H9c2 cells. Hypoxia in turn is associated with
ischaemia as a result of myocardial infarction/unstable angina, and it may be that this model system can be used to study the effects and role of ER stress in cardiovascular conditions leading to hypoxia.

- Etravirine and rilpivirine treatment led to ER stress in H9c2 cells, loss of cell viability taken as mitochondrial damage and apoptosis through the caspase dependent pathway in the cardiac cells. The underlying mechanism did not appear to include an increase in oxidative stress or overactivation of PARP.

- Etravirine and rilpivirine led to endothelial cell damage \textit{in vitro} and dysfunction possibly mediated through overactivation of PARP and the pro-inflammatory effects of the NNRTIs.

- The second generation drugs etravirine and rilpivirine appear to have less damaging effects than the first generation efavirenz in both endothelial and cardiac cells \textit{in vitro}.

6.3 \textbf{Main limitations}

Limitations of this study include those common to all \textit{in vitro} studies trying to mimic the effects of a disease or toxicity that in clinical situations is only observed following many years. HIV patients on ART can expect an almost normal lifespan and may be taking these drugs for 40-50 years. This study used concentrations of ART drugs between 1 and 100 µM with the highest concentration being supraphysiological and designed to confirm the lack of toxicity if that had been observed, which for abacavir it was. These concentrations were based on previous
work in the laboratory and other published studies on ART drugs. Previous studies on endothelial cells have used concentrations of efavirenz ranging from 1 to 32 µM (Jamaluddin, Lin et al. 2010, Orden, De Pablo et al. 2014, Bertrand and Toborek 2015) and in hepatocytes from 5-50 µM (Blas-Garcia, Apostolova et al. 2010, Bumpus 2011, Apostolova, Gomez-Sucerquia et al. 2013, Blas-Garcia, Polo et al. 2014) and neuronal cells from 1.5 to 50 µM (Blas-Garcia, Polo et al. 2014, Purnell and Fox 2014).

Steady state $C_{\text{max}}$ for the drugs used in this study vary quite widely: 12.6 µM for efavirenz, 2.3 µM for etravirine, 0.81 µM for rilpivirine and 6.9 µM for abacavir. These concentrations are in some cases significantly lower than those that were observed to cause cardiovascular cellular damage in our study. How can this clinical data be reconciled with both our in vitro data presented here and previously published studies?

Firstly, these $C_{\text{max}}$ concentrations are obtained in healthy volunteers and studies in HIV patients have identified wide fluctuations in $C_{\text{max}}$ concentrations being influenced by gender, race and CYP P450 polymorphisms (Burger, van der Heiden et al. 2006). In addition, ART drug $C_{\text{max}}$ concentrations have been shown to vary due to interactions with other medications the patient may be on. E.g. co-administration with omeprazole, a common drug for heartburn, increases rilpivirine $C_{\text{max}}$ by 40%.
Therefore it is not impossible that $C_{\text{max}}$ concentrations of these ART drugs in HIV patients could reach 3-4 times what has been reported in healthy individuals.

Secondly, there is an established dogma that treatment of an in vitro system with high concentrations of a drug can mimic long-term exposure of much lower concentrations of drug in clinical situations. Is this really the case, probably not but does this mean that data from short-term exposures to high concentrations of drug in vitro are not important? The answer to this is most definitely no; data from these experiments provide evidence of underlying pathological issues that can be expanded into clinical studies. For example clinical studies link efavirenz to myocardial infarction without the drug affecting known risk factors such as lipid profile leaving the underlying pathology unknown. Efavirenz has been shown to cause endothelial cell dysfunction in vitro therefore studies measuring endothelial cell function in patients on efavirenz to investigate if that is impaired are now warranted. Our data show rilpivirine lead to endothelial activity impairment in vitro. The drug is a second generation NRTI and as such the clinical population of patients on rilpivirine is relatively low. Additionally, HIV infected individuals prescribed rilpivirine have not been on this drug for an extended period of time, and therefore no cardiovascular toxicity studies have been published. However, our preclinical data suggests that HIV patients on rilpivirine are monitored for signs of cardiovascular disease. It may be that rilpivirine has no cardiovascular damaging
effects in the clinic but the \textit{in vitro} data ensures that the possibility is investigated for the benefit of the patient.

Thirdly, and perhaps most importantly, there may be factors in our \textit{in vitro} system that mean the true concentration of ART drug the cells are exposed to is much lower than that added to the treatment media. Interestingly the range of concentrations of efavirenz used in the published \textit{in vitro} endothelial cell studies seem to reflect the FBS percentage of the treatment media and this may be a critical confounding factor when selecting the most physiological relevant concentration of ART drug to investigate. The studies that observed effects of efavirenz at 1 µM were using serum free media (Bertrand and Toborek 2015) whereas the studies observing effects at the higher end of the concentration range, 25 or 32 µM were using FBS levels from 2-5 % (Jamaluddin, Lin et al. 2010, Orden, De Pablo et al. 2014). All of the ART drugs used in this study bind to plasma proteins such as albumin; efavirenz >99 % bound, rilpivirine 99.7 % bound, etravirine 99.9 % bound, and abacavir 49 % bound. The \( C_{\text{max}} \) values above indicate the concentration of ARTs found in the plasma, though it is unclear from the published literature whether this includes both protein bound and unbound drug, however convention suggests that the concentrations reported are for the total amount of drug in the plasma. The concentrations reported in our study are those in treatment media that contains 3 % FBS, therefore the amount of free drug not bound to protein available to interact with the cells in our system is unknown. In preliminary experiments we found that albumin, a major component of
FBS, was able to provide significant protection against efavirenz-mediated endothelial cell damage. As all NNRTIs are more than 99% bound to protein, it may be the actual concentration of free drug in our system is far closer to the concentration the cells are exposed to in patients on NNRTI therapy than it initially appears.

In hindsight should we have carried out our experiments in serum free media to reduce protein binding and be more certain of the real concentration of drug the cells are being exposed to, well this presents its own challenges particularly for long term experiments as a lack of FBS may deprive the cells of essential survival factors making them even more susceptible to the drug being applied or changing the way they respond to the drug. In addition to making it harder to compare our data with other published ART drug studies in cardiovascular cells, which for the most part have used similar levels of FBS. Maybe measuring free concentration of drug in the treatment media and reporting results based on that would be more appropriate and useful to compare back to clinical data though that may have entailed developing analytical chemistry techniques to obtain accurate concentration data.

Overall, for the reasons outlined above the concentrations of drug used in these studies were appropriate for our overall objective to determine possible cardiovascular toxicity of ART drugs.
6.4 Implications

- These results show that the second generation NNRTIs etravirine and rilpivirine have the same effects on cardiac and endothelial cells and function as the first generation efavirenz, although to a lesser extent, suggesting that they may have a safer cardiovascular side effect profile. The data does however suggest that the second generation NNRTIs still may be linked to an increased risk of cardiovascular cell dysfunction and hence cardiovascular disease, therefore careful clinical monitoring should be implemented in patients prescribed these drugs.

- The results obtained with abacavir, show that abacavir does not enhance the damaging effects of palmitic acid and hypoxia, on a cellular level.

- It has been proposed that protease inhibitors that lead to ER stress could possibly be used in the treatment of cancers in order to induce controlled cell death via apoptosis (Bruning, Burger et al. 2009). A similar role may be possible for NNRTIs identified as ER stress inducers in this study.

- ER stress has been shown to be involved in the development of a range of different diseases. PERK inhibitors have been shown to be beneficial by preventing ER stress associated with the development and progression of neurodegenerative disorders in animals. If the ER stress results seen in the rat myocardial cells are replicated in human cells and found to be a main mechanism causing cardiovascular side effects in HIV patients, there may be a clinical option for PERK inhibitors to prevent these effects.
6.5 Future perspectives

Future studies could be performed to answer some of the questions raised during this study.

6.5.1 Abacavir

The above results show that treatment with abacavir doesn’t cause direct damage to the rat cardiac cell line used in the study, nor does abacavir enhance the damaging effects of hypoxia and fatty acids. Previous work has suggested that abacavir has a highly reactive metabolite that is damaging to cells, causes platelet activation and lead to low grade inflammation in a subset of the population. These theories could be investigated further. Additionally, other underlying mechanisms could be explored as a cause of the increased risk in myocardial infarction. E.g. could perhaps abacavir treatment reduce the number of circulating endothelial progenitor cells involved in the regeneration of the endothelium? Endothelial progenitor cells are crucial in regenerating the monolayer of endothelial cells. A decreased number of endothelial progenitor cells may therefore affect endothelial health. This could perhaps explain why the increased risk in CVD associated with abacavir is reversible on cessation of the drug, and could be explored as a possible underlying mechanism.
6.5.2 NNRTIs

Although some of the damaging mechanisms involved have been investigated in this study, other experiments could be performed to further evaluate the cardiovascular cellular effects of the second generation NNRTIs. In particular, it would be interesting to use more sensitive methods, e.g. MitoSox, to explore the possible involvement of oxidative stress as a pathway leading to cardiovascular damage as well as experiments investigating the pathway leading to the substantial increase in IL-8 following etravirine exposure.

Furthermore, the above experiments were performed in cell lines. The next step could be to replicate the experiments in primary human endothelial and heart cells, and from there investigate and compare the effects of the NNRTIs in animal models. Also, non-invasive studies in humans comparing flow mediated dilation between patients on rilpivirine and efavirenz based regime with the same tenofovir and emtricitabine backbone could be performed, to compare their effects on endothelial function in vivo. Etravirine is reserved for virological failure and therefore not often encountered in clinical practise.

6.6 Conclusions

Our data suggest that the underlying pathological mechanism by which abacavir increases the risk of myocardial infarction in HIV patients is not via a direct toxic effect on the heart. We were unable to show a direct damaging effect of abacavir on
cardiac myocytes and neither were we able to demonstrate that abacavir increases the susceptibility of cardiac myocytes to known damaging agents that increase the risk of myocardial infarction i.e. hypoxia and lipotoxicity. Therefore, it may be either the previously published endothelial cell damaging effects or a currently unknown cellular mechanism of abacavir that causes the increased risk of myocardial infarction.

However, we were able to for the first time demonstrate that second generation NNRTIs rilpivirine and etravirine display cardiovascular cell toxicity, though for the most part at a reduced level compared to the first generation NNRTI efavirenz. This suggests that second generation NNRTIs may clinically have a better but not perfect cardiovascular safety profile and this may become apparent as more patients are prescribed these drugs over first generation NNRTIs and clinical investigations begin to be published.

NNRTIs are first line treatment as a part of the lifelong HAART regimes, a fact that is unlikely to change in the near future. Further improving the side effect profile on the endothelium and myocardium should be a priority in the development of the third generation of this class of drug.
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additional nucleoside reverse transcriptase inhibitors." Antivir Ther 12(7): 1075-1085.


APENDIX

LIST OF ABSTRACTS PRESENTED AT SCIENTIFIC MEETINGS


3. Cardiovascular adverse effects of antiretroviral agents efavirenz and rilpivirine used in treatment of HIV, on EA.hy926 cells and aortic rings. 17th World Congress of Basic and Clinical Pharmacology 2014, Cape Town, South Africa.