A BIOCHEMICAL ASSESSMENT OF STRESS RESPONSE FOLLOWING ACUTE AND PROLONGED EXPOSURE TO ANTIRETROVIRAL DRUGS (NUCLEOSIDE REVERSE TRANSCRIPTASE INHIBITORS) *IN VITRO*

BY

SAVANIA NAGIAH

BMedSci (Hons) Medical Biochemistry

Master of Medical Science

Submitted in the fulfilment of the degree Doctor of Philosophy (Medical Biochemistry), School of Laboratory Medicine and Medical Sciences, College of Health Science, University of KwaZulu Natal

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DECLARATION

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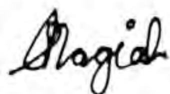

<u>02/03/16</u>

Ms Savania Nagiah **Date**

DEDICATION

To my grandfather, the late Mr T. M. Naidu. All I have learnt of spirituality, the pursuit of knowledge, humility, righteousness and unconditional love has stemmed from the example set by you. Without your influence, I would not have set out on this path, living up to the highest form of myself.

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Department of Medical Biochemistry (2012-2015)

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ABBREVIATIONS

$\Delta\psi_m$	Mitochondrial membrane depolarisation
3TC	Lamivudine
8-oxoG	Guanine 8-oxo-7,8-dihydroguanine
AAA+	ATPases-associated with diverse cellular activities
ABC	Abacavir/ATP-binding cassette
ABCC4	ATP-binding cassette C 4
ADME	Absorption, distribution, metabolism, excretion
ADP	Adenine diphosphate
AIDS	Acquired Immuno-Deficiency Syndrome
AO	Antioxidant
APV	Amprenavir
ARE	Antioxidant response element
ARV	Antiretroviral
ATF6	Activating transcription factor 6
ATP	Adenine triphosphate
ATPase	Adenosine triphosphatase
ATZ	Atazanavir
AZT	Zidovudine
BSA	Bovine serum albumin
bZIP	Basic region leucine zipper
CA	Capsid
CAT	Catalase
CDC	Centre for Disease Control
cDNA	Complementary DNA
CHOP	CCAAT-enhancer-binding homologous protein
CYP	Cytochrome P
d4T	Stavudine
DCF	2',7'-dichlorofluorescein
ddC	Zalcitabine
ddI	Didanosine

DNA	Deoxyribose nucleic acid
DNA pol ɣ	DNA polymerase gamma
eiF2α	Eukaryotic translation initiation factor 2 alpha
Env	Envelope
ER	Endoplasmic reticulum
ETC	Electron transport chain
ETV	Entecavir
EVG	Emtricitabine
FACS	Fluorescence activated cell sorting
FDA	Food and Drug Administration
GAPDH	Glyceraldehyde-3-Phosphate Dehydrogenase
gp	Glycoprotein
GPx	Glutathione peroxidase
GR	Glutathione reductase
GSH	Reduced glutathione
GSSG	Oxidized glutathione
H2DCF-D	2',7'-dichlorodihydrofluorescein
H2DCF-DA	dichlorodihydrofluorescein diacetate
H_2O_2	Hydrogen peroxide
HAART	Highly active antiretroviral therapy
HepG2	Human hepatoma
HIV	Human Immuno-deficiency Virus
HO-1	Heme-oxygenase 1
HRP	Horse-radish peroxidase
HSP	Heat shock protein
IDV	Indinavir
IN	Integrase
IRE-1	Inositol-requiring enzyme 1
JNK	c-Jun N-terminal kinase
LDH	Lactate dehydrogenase
Lon	Lon protease
LPV	Lopinavir

MA	Matrix
MDA	Malondialdehyde
miR-124a	MicroRNA-124a
miRNA	MicroRNA
mt	Mitochondrial
mtDNA	Mitochondrial DNA
MRP	Multi-drug resistance protein
NAD+	Nicotinamide adenine dinucleotide (oxidized)
NADH	Nicotinamide adenine dinucleotide
NBD	Nucleotide binding domain
NC	Nucleocapsid
Nef	Negative factor
NFDM	Non-fat dry milk
NFV	Nelfinavir
NNRTI	Non-nucleoside reverse transcriptase inhibitor
NRF1/2	Nuclear respiratory factor 1/2
Nrf2	Nuclear erythroid 2 related factor 2
NRTI	Nucleoside/nucleotide reverse transcriptase inhibitor
O_2	Oxygen
$O_2^{\bullet -}$	Superoxide anion
OD	Optical density
OGG1	8-oxoG glycosylase
OH•	Hydroxyl radical
$OH_2\bullet$	Hydroperoxyl radical
ORF	Open reading frame
OXPHOS	Oxidative phosphorylation
PBS	Phosphate saline buffer
PCR	Polymerase chain reaction
PERK	Protein kinase RNA-like endoplasmic reticulum kinase
PGC-1α	Peroxisome proliferator-activated receptor gamma coactivator 1-alpha
PI	Protease inhibitor
pol	Polymerase

PR	Protease
Pri-miRNA	Primary transcript microRNA
qPCR	Quantitative polymerase chain reaction
RBI	Relative band intensity
Rev	Regulator of expression of virion proteins
RISC	RNA-induced silencing complex
RLU	Relative light units
RNA	Ribose nucleic acid
ROS	Reactive oxygen species
RT	Reverse transcriptase
RTV	Ritonavir
SDS	Sodium dodecyl sulphate
SIRT	Sirtuin
SQV	Saquinavir
SOD	Superoxide dismutase
Tat	Trans-activator of transcription
TBARS	Thiobarbituric reactive substances
TCA	Tricarboxylic acid
TCEP	Tris-(2-carboxyethyl)-phosphine
TFAM	Mitochondrial transcription factor A
TFV	Tenofovir
TMCII4	Darunavir
TMD	Transmembrane domain
TPV	Tipranavir
TTBS	Tween 20-Tris buffered saline
UCP	Uncoupling protein
UPR	Unfolded protein response
UPR^{mt}	Mitochondrial unfolded protein response
UTR	Untranslated region
Vif	Viral infectivity factor
Vpr	Viral protein R
Vpu	Viral protein U

WHO	World Health Organisation

ABSTRACT

Nucleoside reverse transcriptase inhibitors (NRTIs) are the most extensively used antiretroviral (ARV) drugs in highly active antiretroviral therapy (HAART). The long term use of HAART is associated with changes to metabolic parameters leading to lipodystrophy and metabolic syndrome, as well as toxicity to high energy demand organs e.g. liver, kidney, heart, and nervous system. Underlying the myriad of NRTI-associated adverse health outcomes is mitochondrial (mt) toxicity. Although inhibition of mtDNA synthesis was one of the first identified mechanisms of toxicity, it did not provide a holistic explanation for all NRTIs. Furthermore, variations in adaptive stress responses were observed following acute and chronic exposure to NRTIs. Insight gained from the molecular changes induced by NRTIs will enable effective management and limit adverse health outcomes.

The human hepatoma (HepG2) cell line was used as an *in vitro* model to investigate changes to mt function, cellular redox status, and antioxidant response following acute [24 hour (h)] and prolonged (120 h) exposure to NRTIs – Zidovudine (AZT, 7.1µM); Stavudine (d4T, 4µM); Tenofovir (TFV, 1.2µM). Long term exposure to AZT and d4T reduced mtDNA levels (120h, AZT: 76.1%; d4T:36.1%, $p<0.05$) and mt function was compromised as evidenced by reduced ATP levels (AZT: 38%; d4T: 56.4%) and increased mt membrane depolarisation ($p<0.02$). Tenofovir compromised mt function at 120 h independently of depleting mtDNA levels. Oxidative stress parameters were significantly elevated by AZT and TFV at 24h; and all NRTIs at 120 h ($p<0.05$). Endogenous antioxidant response was highest in TFV in both time periods (120h; $p<0.05$).

Once NRTI induced oxidative stress in HepG2 cells was established, protein homeostatic response to oxidative stress was investigated. Lon protease expression and related endoplasmic reticulum (ER) stress was evaluated. The data showed that ATP-dependent protein homeostasis responses Lon, heat shock protein 60 (HSP60) and ER stress were significantly increased at 24 h (>2 fold); but significantly decreased at 120 h for all NRTIs ($p<0.005$).

The compromised ATP-dependent stress response then led to the assessment of an ATP-dependent drug transporter responsible for efflux of xenobiotics in hepatocytes. The transporter, ATP-binding cassette C4 (*ABCC4*), is regulated by microRNA (miR-) 124a. Regulation of *ABCC4* by miR-124a has implications for bio-accumulation and resultant toxicity. An inverse relationship between miR-124a and *ABCC4* mRNA levels in all treatments at both time periods was observed. All NRTIs elevated miR-124a levels at 24 h ($p=0.0009$) and this observation was consistent in d4T and TFV treated HepG2 cells at 120 h ($p<0.0001$). This was accompanied with a concomitant decline in *ABCC4* mRNA levels ($p<0.0001$) relative to the control. Prolonged exposure to AZT caused a decrease in miR-124a and elevated *ABCC4* mRNA levels. Protein expression of multi-drug resistance protein 4 (MRP4), coded for by *ABCC4,* did not correlate to mRNA expression. At 120 h, all NRTIs

caused significant depletion of MRP4 (possibly due to oxidative cell membrane damage or ATP depletion).

In conclusion, all three NRTIs compromised mt function and induced oxidative damage in HepG2 cells, with greater toxicity over 120 h. Reduced ATP levels compromised the ATP-dependent stress response proteins and xenobiotic detoxification. Tenofovir could be considered a safer alternative as it elicited the highest antioxidant response in spite of reduced mt function.

CHAPTER ONE

INTRODUCTION

Human immuno-deficiency virus (HIV), the causative agent of Acquired Immuno-Deficiency Syndrome (AIDS), is a retrovirus that progressively impairs the immune system. The immuno-suppression observed in AIDS increases the risk of often fatal opportunistic infections. The first clinical observation of AIDS was made in 1981 in a group of homosexual intravenous drug users (CDC, 1982), and the AIDS epidemic officially began in June 1982. As of 2013, it is estimated that there are 35 million people infected with HIV globally (UNAIDS, 2013). In South Africa, approximately 12% of the population are HIV positive (Simbayi et al., 2014). Comparatively, HIV/AIDS is the 5th leading cause of death, and the leading communicable disease related cause of death in the world (WHO, 2014). The socio-economic impact of HIV/AIDS has pushed this disease to the forefront of clinical and molecular research.

The advent of anti-HIV treatment was one of the most significant advancements in modern medicine. According to UNAIDS, there were an estimated 11.7 million people on antiretroviral (ARV) therapy in 2013 (UNAIDS, 2013). Prior to the 1990's, the only treatment available was the management of AIDS related opportunistic infections. In 1991 the first ARV drug against HIV, Zidovudine (AZT), was approved by the Food and Drug Administration (FDA). Today, there are twenty four ARV drugs that are FDA-approved for HIV treatment. Antiretroviral therapy works on the principle of inhibiting the viral life cycle. This prevents the replication of mature, infectious virus, thus reducing viral load.

Antiretroviral drugs are categorized according to the phase of the viral life cycle that they target. There are six major classes of ARV drugs: nucleoside/nucleotide reverse transcriptase inhibitors (NRTIs), non-nucleoside reverse transcriptase inhibitors (NNRTIs), protease inhibitors (PIs), integrase inhibitors and fusion/entry inhibitors. The most effective form of therapy is a combination of various classes of drugs. First line therapy usually includes two NRTIs and one PI or NNRTI. Triple drug therapy, known as highly active antiretroviral therapy (HAART), is a potent and effective therapy in suppressing viral load (Arts and Hazuda, 2012).

The incidence of opportunistic infections due to immuno-suppression has declined in HIV positive patients on HAART, with noticeably decreased mortality. The chronic nature of anti-HIV treatment, however, has seen the emergence of long term side effects. Adverse side effects lead to lack of adherence in patients, increasing the risk of viral drug resistance (Duran et al., 2001). Toxicity depends on a variety of factors including exposure time, drug concentration, pharmacokinetics, and tissue specificity. Although individual drugs have varying toxicities, class specific health outcomes have been observed over time (Montessori et al., 2004). Protease inhibitors, for example, have been associated with insulin resistance leading to type 2 diabetes mellitus; and altered fat metabolism favouring dyslipidaemia and lipodystrophy (Carr et al., 1998). Rashes and lipid disorders are common

outcomes of NNRTI use (Reust, 2011). Nucleoside reverse transcriptase inhibitors appear to have toxic profiles reminiscent of mitochondrial (mt) diseases (Kohler and Lewis, 2007, Montaner et al., 2003).

Nucleoside reverse transcriptase inhibitors are the most extensively used class of anti-HIV drugs. These drugs are the cornerstone of HAART and are also used in non-infected individuals as pre-exposure prophylaxis (Celum and Baeten, 2012, Service, 2014). The cost effectiveness of NRTIs Lamivudine (3TC) and Stavudine (d4T) make these drugs the basis of first line therapy in developing countries (Subbaraman et al., 2007). This standardized approach to ARV therapy doesn't take into account drug interactions in individual patients. Under these circumstances, drug toxicity and adverse side effects are common occurrences. The most pressing side effects related to NRTI use are changes to fat metabolism, favouring lipodystrophy and metabolic syndrome, hepatic steatosis, peripheral neuropathy, hyperlactatemia, lactic acidosis, pancreatitis, cardiomyopathy and peripheral myopathy (Carr and Cooper, 2000, Moyle, 2000).

Underlying the myriad of adverse health outcomes associated with long term NRTI use is compromised mt function (Brinkman et al., 1998). Thymidine analogues, including AZT and d4T, cause severe depletion of mtDNA by inhibition of the enzyme DNA polymerase (pol) ɣ (Kakuda, 2000). Depletion of mtDNA causes compromised biosynthesis of components of the electron transport chain (ETC). Consequently, cellular energy status, redox balance and mt regulated metabolism is disrupted.

Stavudine is considered a mt toxicant, but is still used in developing countries due to its cost effectiveness. A strong link between lipodystrophy, depleted mtDNA in adipose tissue, and d4T use has been established (van der Valk et al., 2004, McComsey et al., 2005, Shikuma et al., 2001). Chronic use of d4T-containing regimens has also been associated with pancreatitis, lactic acidosis and hepatic steatosis (Miller et al., 2000, Makinson et al., 2008, Bolhaar and Karstaedt, 2007). When toxicity presents itself, d4T is usually substituted with Tenofovir (TFV) or AZT (Castelnuovo et al., 2008, Gallant et al., 2004, Domingo et al., 2004). Substitution of d4T with TFV improves dyslipidaemia, subcutaneous fat and mt toxicity profiles (Domingo et al., 2004, Ribera et al., 2008). Although TFV exhibits lower mt toxicity in adipose tissue, mt toxicity has been identified as a cause of renal failure with TFV use (Kohler et al., 2009). Zidovudine also exhibits mt toxicity leading to myopathy and cardiomyopathy (Dalakas et al., 1990, Tanuma et al., 2003). Although d4T, AZT and TFV exhibit varying toxicity profiles in different tissues, target tissues are all high energy demand and rich in mitochondria. Compromised mt function is not always correlated to mtDNA levels, hence new avenues of mt toxicity mechanisms need to be investigated.

The liver is the metabolic hub of the body and is responsible for chemical modification of xenobiotics, aiding in excretion. Due to the high level of drug metabolising enzymes, hepatocytes bear the brunt of

oxidative insult and xenobiotic accumulation (Park et al., 1995). Cytoprotective stress response are imperative to maintaining homeostasis and preventing drug induced liver damage. The antioxidant defence and mt biogenesis regulators are the major components of cytoprotection during mt toxicity. The master regulator of the antioxidant response, nuclear-erythroid 2-related factor 2 (Nrf2), translationally regulates a battery of antioxidant defence genes (Nguyen et al., 2009). This transcription factor has already been recognised for its protective role in drug induced cytotoxicity (Copple et al., 2008). Furthermore, Nrf2 induces the expression of mt biogenesis genes, overlapping its function with peroxisome proliferator-activated receptor gamma co-activator 1 alpha (PGC-1α) (Piantadosi et al., 2008). An increase in mt biogenesis markers is a compensatory mechanism in mt disease and toxicity. Both PGC-1α and Nrf2 are central to maintaining mt function and antioxidant response in mt rich tissue, including the liver (Itoh et al., 1997, Finck and Kelly, 2006).

The clearance of proteins subject to oxidative damage is also an important molecular mechanism in NRTI toxicity. Inability to clear oxidatively modified proteins, and particularly mt proteins, is characteristic of biological aging (Farout and Friguet, 2006, Bulteau et al., 2006). Chronic use of NRTIs has been related to accelerated aging and age-associated disorders (Smith et al., 2012). Compromised proteolytic clearance of damaged proteins during NRTI-induced oxidative stress is a likely contributor to the observed conditions. The mt matrix protein Lon protease (Lon) is the main protease responsible for maintaining mt protein homeostasis (Gur and Sauer, 2008). Lon works in conjunction with the endoplasmic reticulum (ER) stress response to mediate proteolytic clearance of misfolded proteins and protein occlusions. To date only one study has evaluated *Lon* mRNA expression in relation to HAART (Pinti et al., 2010). The study showed up-regulation of *Lon* in adipose tissue of patients on HAART presenting with lipodystrophy. This implicates Lon as a stress response to NRTI toxicity.

Alternative detoxification mechanisms may also be provided by cellular drug transporters which function to reduce the intracellular concentration of NRTIs, reducing bioaccumulation. In this regard, the ATP-binding cassette (ABC) family of transporters facilitates cellular export of NRTIs in liver cells (Keppler, 2011). These transporters are known to be induced by Nrf2, implicating these membrane proteins in stress response (Aleksunes et al., 2008). Furthermore, TFV and AZT have been identified as substrates for *ABCC4*, a member of the ABC family. Differential regulation of *ABCC4* in hepatocytes will influence the intracellular concentration of NRTIs, having implications for toxicity. Epigenetic regulation of drug transporters is a relatively new field of research linking microRNA (miRNA) to drug resistance/toxicity (He et al., 2015). MicroRNAs are small non-coding RNA that cause post-translational repression. These regulators are highly relevant to ABC transporters, with various miRNAs targeting multiple ABC transporters (Haenisch et al., 2014). The regulation of ABC transporters by miRNA has not been investigated with NRTI toxicity.

We hypothesized that NRTIs (AZT, d4T and TFV) induced biochemical stress responses following acute (24 h) and prolonged (120 h) exposure at maximum plasma level concentrations in human hepatoma (HepG2) cells.

In order to test the hypothesis we measured cytoprotective effects involved in mt function, oxidative stress, protein homeostasis and epigenetic regulation of cellular detoxification in HepG2 (liver) cells. The specific research objectives were assessed as follows:

- mitochondrial and oxidative stress response following acute and prolonged exposure to NRTIs
- ATP-dependent protein maintenance networks in response to acute and prolonged NRTI-induced oxidative stress
- differential regulation of ATP-dependent efflux drug transporter (*ABCC4*) by microRNA-124a following acute and prolonged exposure to NRTIs

CHAPTER TWO

LITERATURE REVIEW

2.1. Human Immuno-Deficiency Virus

2.1.1. HIV morphology

The structural biology and biochemistry of HIV has been a focus point for therapeutic intervention. The virus belongs to the lentivirus genus, a subgroup of the family *Retroviridae*. Unlike most viruses, HIV is spherical in structure, with a diameter of 100-120nm (Sierra et al., 2005). There are two identified HIV strains: - HIV-1 and HIV-2. HIV-1 is more virulent and is the more commonly observed strain globally. HIV-2 has a much lower virulence/infectivity and is isolated to areas of West Africa.

The HIV genome consists of two identical 9.2kb single RNA strands (Sierra et al., 2005). The genome contains three open reading frames (ORF) for structural proteins. Group specific antigen (*Gag)* encodes for all structural proteins [matrix (MA), capsid (CA) and nucleocapsid (NC)]. The polymerase (*pol)* ORF encodes for all viral enzymatic components [protease (PR), reverse transcriptase (RT), integrase (IN), ribonuclease]. Finally, the envelope (*Env)* ORF encodes viral envelope proteins [glycoprotein (gp)120, gp41] (Wilk and Fuller, 1999). The HIV genome encodes for a further six accessory genes involved in the regulation of viral replication: Trans-activator of transcription (*Tat)*, regulator of expression of virion proteins (*Rev)*, negative factor (*Nef)*, viral infectivity factor (*Vif)*, viral protein R (*Vpr)*, and viral protein U (*Vpu)*.

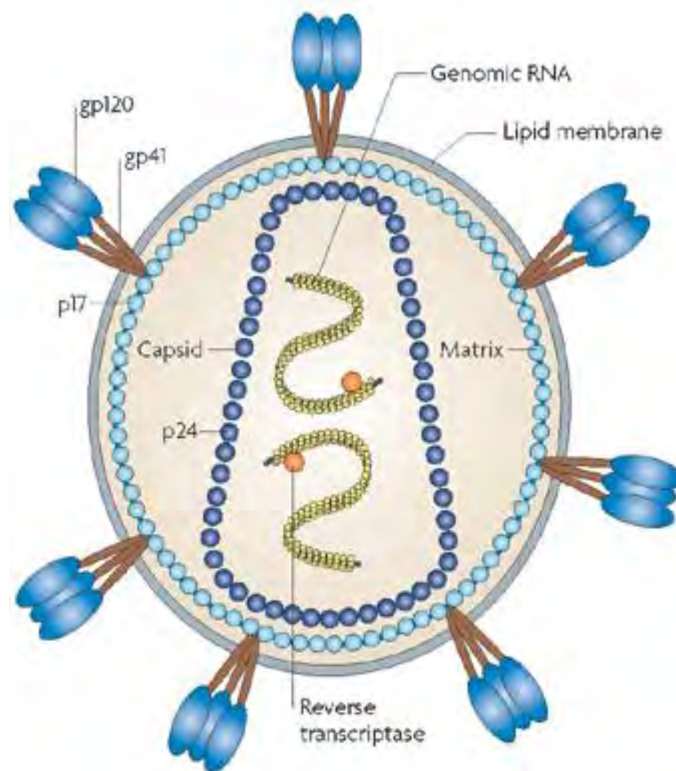

Figure 2.1: Diagram of HIV mature virion. The lipid bi-layer is derived from the host cell. The structural proteins forming the core are matrix protein (p17), capsid protein (p24), and nucleocapsid (p7). The capsid contains the two strands of viral RNA and viral enzymes including reverse transcriptase (Karlsson Hedestam et al., 2008)

The virion is enveloped in a bi-layer of phospholipids that are of cellular origin. On the viral envelope are protein protrusions which facilitate binding to the host cell. The protrusions are anchored by the transmembrane protein, gp41. Viral protein gp120 projects out of the viral envelope to facilitate attachment to the host cell and subsequent fusion. Enclosed in the viral envelope is the viral MA, consisting of viral protein p17, which maintains the structural integrity of the virion. At the viral core is the CA. The CA is conical and is constituted of viral protein p24. Within the CA, two strands of viral RNA are tightly bound to p7, the viral NC protein. Also contained in the NC are the enzymes required for viral replication: RT, IN, PR (Ganser-Pornillos et al., 2008, Sierra et al., 2005, Turner and Summers, 1999).

2.1.2. Viral replication

HIV primarily targets immune cells such as $CD4^+$ helper T cells, macrophages, dendritic cells and microglial cells. The CD4 receptor on the immune cell is a docking site for gp120. Once bound to the host cell, gp120 undergoes further interaction with chemokine co-receptors, particularly CXCR4 and CCR5 (Clapham and Weiss, 1997, Doranz et al., 1996, Feng et al., 1996). These receptors are determinants of the tropism of HIV. This interaction causes a conformational change that exposes the

hydrophobic domain of gp41, allowing fusion with the host cell. An uncoating event takes place, releasing the CA, containing viral genomic material and enzymes, into the cytosol of the host cell (Figure 2.2).

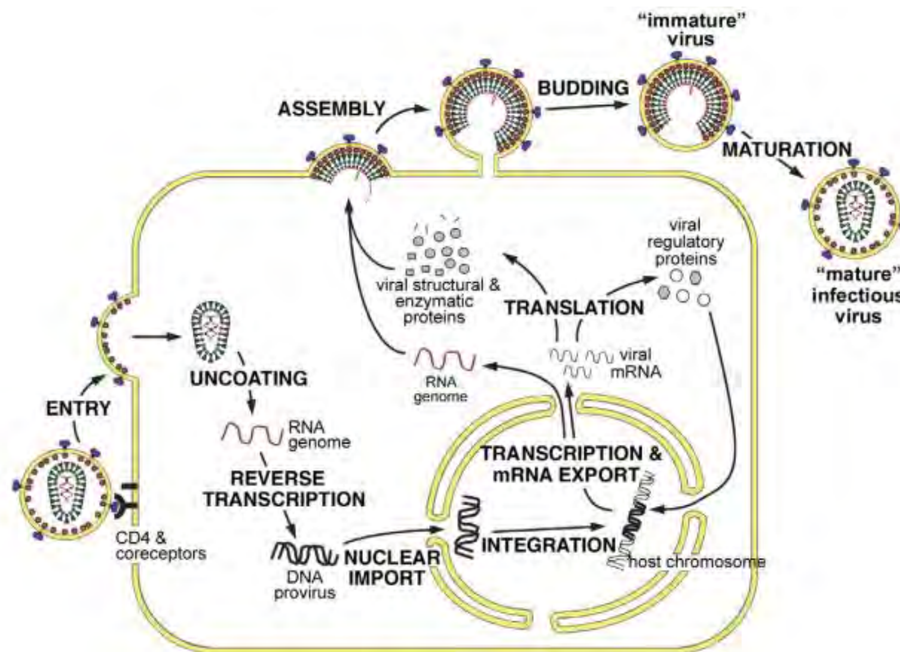

Figure 2.2: Viral replication of HIV-1 (Ganser-Pornillos et al., 2008)

Upon entry into the cytosol, viral RNA is reverse transcribed by RT, forming the viral pre-integration complex. This process is thought to be regulated by accessory protein *Vif* (Turner and Summers, 1999). Nuclear localization of the viral DNA is an active process regulated by MA, IN, RT and *Vpr* (Bukrinsky et al., 1993, von Schwedler et al., 1994). Integration of viral and host DNA takes place in the nucleus. The viral enzyme IN mediates three sequential processes for integration into the human chromosome. Firstly the assembly of viral DNA, secondly endonucleolytic processing of the 3' end of viral DNA, and finally the joining of the strand to cellular DNA (Arts and Hazuda, 2012). This process results in the production of unspliced and spliced mRNA transcripts. The short spliced RNA species encode for *Tat, Rev* and *Nef*. Regulatory protein *Tat* is vital for transcriptional activation. The full length or singly spliced viral RNA contains the introns encoding for *Gag* and *Pol*. These need to be exported to the cytosol for synthesis to occur. The export of the viral RNA is regulated by *Rev*, which binds to the Rev-response element. The formation of this complex recruits protein exportin 1, allowing transport of the viral DNA through a nuclear pore (Ohno et al., 1998).

The viral spike gp120 is derived from gp160, a genomic derivative of *Env*, and is synthesized in the endoplasmic reticulum (ER). From the ER the polyprotein gp120 is transported through the Golgi apparatus and cleaved by furin to gp120 and gp41. These processed proteins, together with *Gag*, *Gag-Pol*, and the viral RNA aggregate at the plasma membrane, enabling the assembly phase. The virion

then begins to bud off the host cell (Figure 2.2). The *Gag* polyproteins will still need to be further cleaved by PR. Once all the structural components have assembled a mature, infectious virion is formed. Mature virus is capable of entering $CD4^+$ cells and utilizing the cellular machinery for viral replication.

Inevitably, the $CD4^+$ T cell pool is significantly depleted as viral replication is sustained. The majority of $CD4^+$ cell loss is due to apoptotic cell death (Gougeon et al., 1996). The progressive decline in immune cells compromises both innate and adaptive immunity, eventually increasing susceptibility to opportunistic infections. Commonly observed health outcomes include Kaposi sarcoma, lymphoma, and infection with tuberculosis, bacterial pneumonia, Cryptococcus, herpes simplex, cytomegalovirus, (Kaplan et al., 2009). In order to maintain $CD4^+$ T cells at a sustainable level to confer protection, viral replication is a key target in reducing the infectivity of HIV.

2.2. HIGHLY ACTIVE ANTIRETROVIRAL THERAPY (HAART)

2.2.1. Nuclease Reverse Transcriptase Inhibitors

Nuclease reverse transcriptase inhibitors (NRTIs) were the first class of drugs approved by the Food and Drug Administration (FDA) for HIV treatment. These drugs form the backbone of highly active antiretroviral therapy (HAART). This class of drugs functions to inhibit viral RT in a competitive fashion. All NRTIs are designed as analogues of native nucleosides/nucleotides.

Table 2.1: List of FDA approved NRTIs used in HAART (WHO, 2010)

Drug Name	Trade Name	Functional Analogue
Abacavir (ABC)	Ziagen	Guanosine
Emtricitabine (EVG)	Emtriva	Cytidine
Didanosine (ddI)	Videx	Guanosine
Lamivudine (3TC)	Epivir	Cytidine
Stavudine (d4T)	Zerit	Thymidine
Zalcitabine (ddC)	Hivid	Pyrimidine
Entecavir (ETV)	Baraclude	Guanosine
Zidovudine (AZT)	Retrovir	Thymidine
Tenofovir (TFV)	Vired	Adenosine

Reverse transcriptase is responsible for the conversion of single stranded viral RNA to double stranded DNA upon entry of the virus into the host cell. Viral transcription utilizes cellular native nucleotides for strand synthesis. Antiretroviral (ARV) drugs belonging to the class NRTI lack the 3’-

OH group at the sugar moiety of the base. Integration of the NRTI during transcription results in chain termination, thus preventing full length viral DNA strand synthesis (Arts and Hazuda, 2012). All NRTIs are administered as pro-drugs. They require addition of a triphosphate group by cellular kinases to become active. Tenofovir (TFV), however, requires the addition of a diphosphate group in order to be activated.

2.2.2. Non-nucleoside Reverse Transcriptase Inhibitors

Non-nucleoside reverse transcriptase inhibitors (NNTRIs) are a chemically diverse class of ARV drugs that also target viral RT. They are primarily metabolized by the enzymes cytochrome P (CYP) 3A4 and CYP1B1, belonging to the CYP450 family. These are used alongside NRTIs to reduce the chances of viral resistance, and may have synergistic anti-viral activity (Basavapathruni et al., 2004).

Unlike NRTIs, the mechanism of inhibition is non-competitive. Furthermore this class of drugs is specific to HIV-1, and is unable to inhibit the replication of other lentivirus, including HIV-2 (De Clercq, 1998). The mechanism of action in NNRTIs is not unlike that of NRTI therapy, in that both manipulate the viral enzyme, RT. However, the mode of action differs as NNRTIs do not bind to the active substrate docking site of the enzyme. These drugs bind covalently to a site proximal to the active site (Figure 2.3). The NNRTI induces the formation of a hydrophobic pocket, changing the structural configuration of RT. This prevents the substrate (native nucleotides) from docking at the active site of RT, resulting in the termination of viral DNA strand synthesis (de Béthune, 2010, De Clercq, 1998).

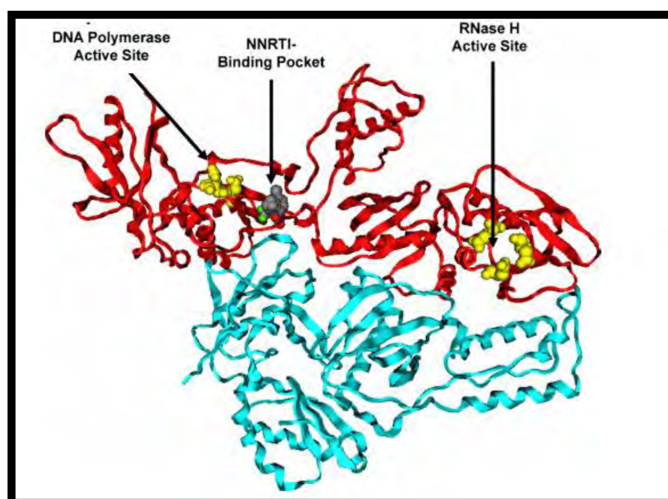

Figure 2.3: Ribbon representation of reverse transcriptase (RT) complex with NNRTI. Red represents viral subunit p66 and blue indicates subunit p51. Image adapted from Sluis-Cremer and Tachedjian, 2008 (Sluis-Cremer and Tachedjian, 2008)

As of 2013 the following FDA approved NNRTIs are available globally: Etravine (Intelence), Delavirdine (Rescriptor), Efavirenz (Sustiva), and Nevirapine (Viramune). Rilpivirine (Edurant) is in phase 3 of clinical trials.

2.2.3. *Protease Inhibitors*

Protease inhibitors (PIs) are considered the most potent anti-HIV agents. However, they also display many side effects. These drugs target the late phase of the viral replication cycle, by inhibiting viral PR. Protease functions to cleave *Gag* and *Gag-Pol* polypeptide precursors during viral maturation (Park and Morrow, 1993). Inhibition of this enzyme prevents the formation of mature, infective virus. There are currently 10 PIs approved by the FDA, as listed in Table 2.2.

Table 2.2: List of FDA approved Protease Inhibitors for HIV treatment (WHO, 2010)

Drug Name	Abbreviation	Trade Name
Amprenavir	APV	Agenerase
Atazanavir	ATZ	Reyataz
Darunavir	TMCII4	Prezista
Fasamprenavir	-	Lexiva
Indinavir	IDV	Crixivan
Lopinavir	LPV	Aluvia
Rotinavir	RTV	Norvir
Lopinavir+Rotinavir	-	Kaletra
Nelfinavir	NFV	Viracept
Saquinavir	SQV	Fortovase/Invirase
Tipranavir	TPV	Aptivus

2.2.4. *Entry inhibitors*

Entry inhibitors consist of two distinct classes – fusion inhibitors and CCR5 antagonists.

2.2.4.1. CCR5 antagonists

This branch of drugs functions as allosteric inhibitors of viral entry. CCR5 is one of the main co-receptors utilized by gp120 to initiate the fusion step during viral replication. Antagonists of CCR5 are generally small molecules that occupy the hydrophobic pockets between the helices of the CCR5 receptor. The presence of these small molecules results in a conformational change in the CCR5 receptor, making it unrecognisable to the virus. The receptor cannot be used to gain entry into the cell. There are currently 3 CCR5 antagonists on the market: Aplaviroc, Vicriviroc and Maraviroc (Dorr et al., 2005).

2.2.4.2. Fusion inhibitors

The fusion phase of the viral life cycle requires the interaction of two domains of the gp41 transmembrane protein – Heptade repeat 1 and 2. These two motifs bind to each other, creating a hairpin structure, pulling the cell membrane toward the viral membrane. Fusion inhibitors mimic one of these domains and interrupt the intra-molecular interaction (Lalezari et al., 2003). These drugs also sensitize the virus to antibodies specific against gp41, as they prolong the exposure time during fusion.

2.2.5. Integrase inhibitors

Integrase processes the 3' end of viral DNA and facilitates strand joining of viral DNA to host DNA. Integrase inhibitors are the newest discovery in targeted ARV therapy (Espeseth et al., 2000, Hazuda et al., 2004). Raltegravir, the first integrase inhibitor, was approved in 2007. Other integrase inhibitors are still in the clinical trial phase (Sato et al., 2006, Shimura and Kodama, 2009). These drugs inhibit viral DNA strand transfer by binding to the complex between viral RNA and IN. Integrase inhibitors possess a metal-binding pharmacophore, enabling the drug to interact with magnesium ion co-factors which are essential for IN function.

2.3. ANTIRETROVIRAL DRUGS AND ADVERSE HEALTH OUTCOMES

2.3.1. Clinical outcomes

2.3.1.1. Lipodystrophy

Lipodystrophy is a condition characterized by loss of subcutaneous adipose in the face, limbs and buttocks, with abdominal and visceral accumulation of fat. Dorso-cervical fat accumulation referred to as "buffalo hump" or "fat pad" is also a symptom affiliated with lipodystrophy. Lipodystrophy is observed in a subset of patients on HAART. Patients with lipodystrophy need not present with all the above mentioned clinical outcomes. Both NRTIs and PIs are associated with the development of lipodystrophy (Carr et al., 2000, Villarroya et al., 2010, Tsiodras et al., 2000). HIV infection also plays a role in the pathogenesis of the disorder (Riddler et al., 2003).

The molecular mechanism leading to the physiological outcomes of lipodystrophy is attributed to the active lipolysis of subcutaneous fat in peripheral regions. Stavudine (d4T)-containing therapy has a strong correlation with the onset of lipodystrophy. A proposed mechanism in this instance is adipose specific mitochondrial (mt) toxicity (Shikuma et al., 2001, McComsey et al., 2005, van der Valk et al., 2004). Excessive lipolysis leads to elevated free fatty acids in circulation and the deposition of fat in visceral regions such as the stomach, back and behind the neck (Villarroya et al., 2005). The elevation of circulating free fatty acids, particularly triglycerides, has metabolic repercussions. Insulin

resistance, elevated total cholesterol, heightened low density lipoprotein cholesterol, and reduced high density lipoprotein cholesterol are observed as consequences of lipodystrophy. Such metabolic outcomes are reminiscent of metabolic syndrome. These factors increase the risk of developing type 2 diabetes and cardiovascular disease (Estrada et al., 2006, Villarroya et al., 2010).

2.3.1.2. Hepatic steatosis

Hepatic steatosis, or fatty liver, is a condition whereby the abnormal retention of lipids causes the formation of triglyceride-containing vacuoles in the liver. Hepatic steatosis is a consequence of disrupted fat metabolism (Reddy and Rao, 2006). The environment created in the event of steatosis promotes inflammation, increasing the risk of hepatitis. Nuclease reverse transcriptase inhibitors: d4T, Zidovudine (AZT) and Didanosine (ddI), have been associated with this condition. The pathological mechanism by which these drugs cause hepatic steatosis is via mt toxicity and consequently disrupted lipid metabolism (McGovern et al., 2006, Sulkowski et al., 2005). A study conducted by Sulkowski et. al in 2005 in a cohort of 137 male HIV patients found 40% of HIV and hepatitis C co-infected men on extensive ARV therapy presented with hepatic steatosis. Stavudine was recognised as a modifiable risk factor in this study. Another study by Sutinen et. al (2002) showed hepatic fat accumulation in patients with HAART-associated lipodystrophy may play a causative role in insulin resistance (Sutinen et al., 2002).

2.3.1.3. Hyperlactataemia

Lactic acidosis is a rare but potentially fatal outcome of NRTI use. Chronic elevated levels of lactic acid is not unusual for HIV infected individuals on HAART (John et al., 2001). Lactic acidosis only presents itself following long term use of NRTIs. The proposed mechanism by which NRTIs increase lactate levels is the inhibition of the enzyme responsible for mtDNA replication, DNA polymerase (pol) ɣ (Figure 2.4). Lactate is a product of anaerobic glycolysis. In the event of mt dysfunction, compromised aerobic respiration shunts pyruvate into lactate production. Long term use of NRTIs, especially strong inhibitors of DNA pol ɣ, leads to the progressive depletion of mtDNA. The result is a reduction in the proteins coded for by mtDNA and subsequent disruption of mt function. During normal glycolysis glucose is converted to pyruvate in the cytoplasm. Pyruvate then enters the mitochondria, where it is converted to Acetyl coenzyme A, which in turn enters the tricarboxylic acid (TCA) cycle. The product of the TCA cycle is NADH, which is utilized in ATP production during oxidative phosphorylation (OXPHOS). When mt function is disrupted, the utilization of pyruvate by mitochondria is compromised. This allows pyruvate to accumulate in the cytoplasm, eventually being converted to lactate by lactate dehydrogenase (LDH).

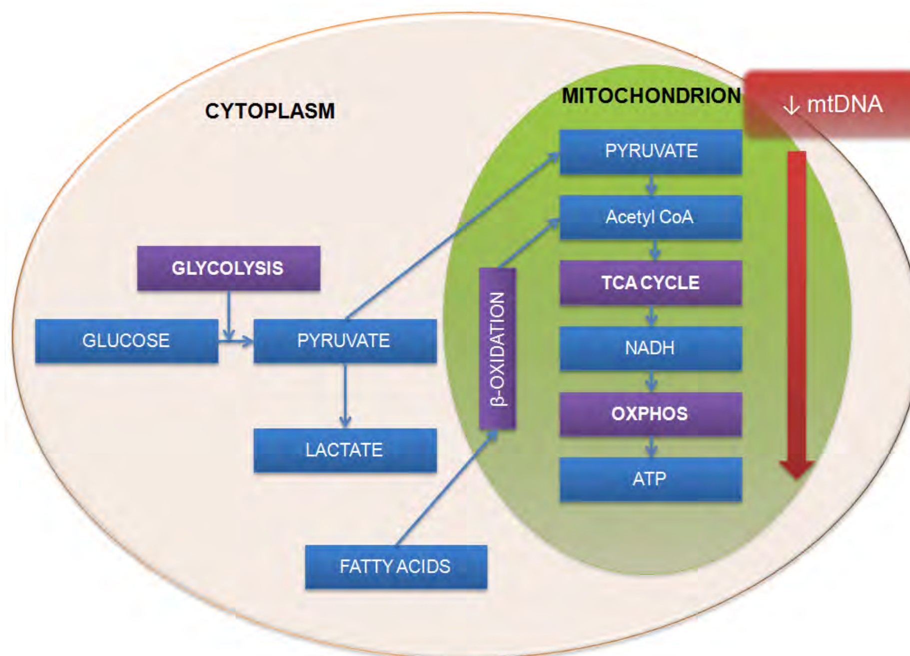

Figure 2.4: Mechanism by which NRTI-associated mitochondrial toxicity causes lactic acidosis (Figure by author)

Lactic acidosis is usually accompanied by hepatic steatosis, further implying mt dysfunction as a causative mechanism. Females on HAART are at higher risk of developing lactic acidosis and hyperlactataemia as compared to males (Bolhaar and Karstaedt, 2007). Furthermore, uninfected infants exposed *in utero* to ARV drugs also display elevated levels of lactate (Alimenti et al., 2003). Previous work done by Montaner et. al in 2003 showed reduced mtDNA preceded the elevation of venous lactate levels in patients on HAART (Montaner et al., 2003).

2.3.2. Biochemical outcomes

2.3.2.1. Mitochondrial Toxicity

The mitochondrion is the metabolic hub of the cell and integrates vital multi-cellular pathways (Figure 2.5). The varied side effects of NRTIs are all linked to disrupted mt function. Mitochondrial toxicity provides a single explanation for a myriad of adverse clinical outcomes as shown in Figure 2.6. Mitochondria-rich tissue with high energy demand (cardiac, hepatic, neuronal, renal) display increased susceptibility to NRTI toxicity (Apostolova et al., 2011). Mitochondrial-driven physiological processes, particularly lipid metabolism, are altered with NRTI use (Carr et al., 2000). Mitochondrial DNA replication and transcription, mt biogenesis, protein synthesis, mt membrane integrity, mt matrix metabolism and ATP production have all been identified as possible targets for NRTI-associated mt toxicity (Apostolova et al., 2011).

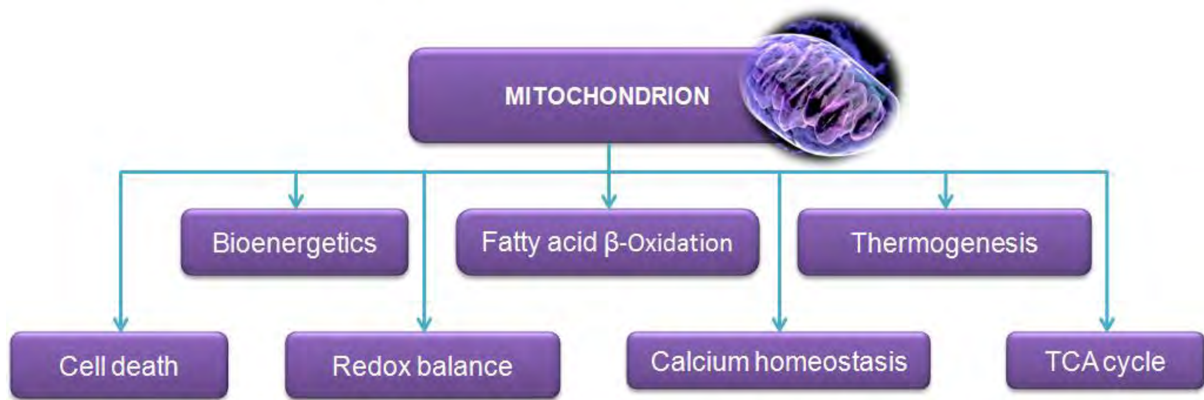

Figure 2.5: Schematic representation of physiological functions of mitochondria (adapted from Apostolova, Blas-García et al. 2011). Mitochondrial toxicants, such as NRTIs, can have far reaching consequences as mitochondrial dysfunction has implications for all the above mentioned processes

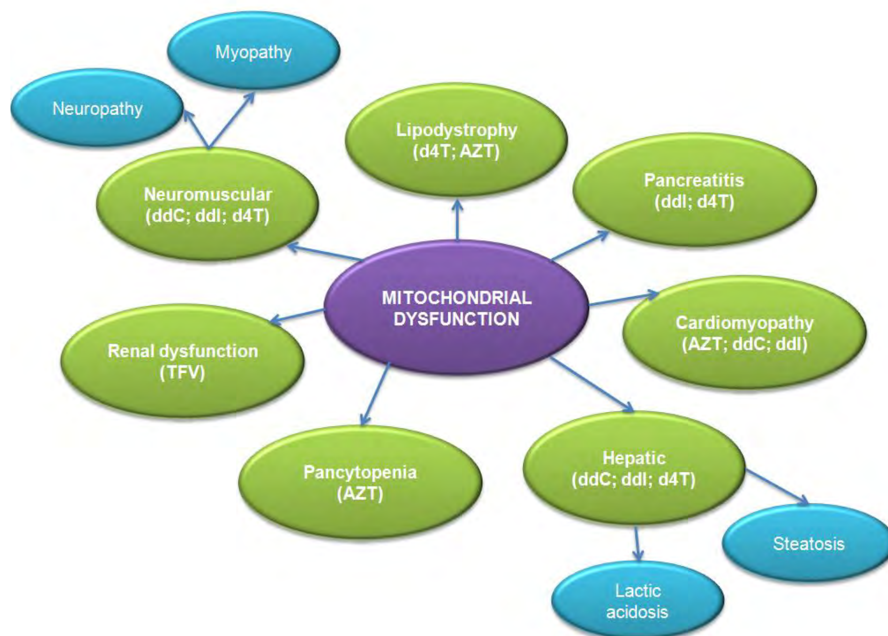

Figure 2.6: Schematic representation of clinical outcomes in NRTI-associated mt toxicity. Individual ARVs display tissue specific toxicity. AZT: Zidovudine; ddC: Zalcitabine; ddI: Didanosine; d4T: Stavudine; TFV: Tenofovir (Figure by author)

2.3.2.2. DNA polymerase gamma hypothesis

The identification of NRTI associated mt toxicity led to the interrogation of this pathological mechanism. One of the earliest theories relating NRTI exposure to mt dysfunction was the "DNA pol

ɣ hypothesis." Aside from being a substrate for viral RT, NRTIs also serve as substrates for endogenous polymerases. Of the five human polymerase enzymes, DNA pol ɣ is the sole enzyme responsible for mtDNA replication and has a high affinity for specific (thymidine analogues) NRTIs (Kakuda, 2000). Incorporation of NRTIs during mtDNA replication results in chain synthesis termination.

Mitochondria have their own DNA distinct from nuclear DNA. Mitochondrial DNA is circular and codes for 13 proteins. Proteins coded for by mtDNA are essential subunits in the electron transport chain (ETC) and are required for efficient OXPHOS. Chronic exposure to NRTIs results in depleted mtDNA levels (Lewis and Dalakas, 1995).Theoretically, depleted mtDNA levels compromise the functionality of the ETC and OXPHOS, resulting in deficient ATP production, mt membrane depolarisation and an elevation in the production of reactive oxygen species (ROS).

Each NRTI has an individual toxicity profile; with dideoxynucleotide analogues [d4T; Zalcitabine (ddC); ddI] and AZT having the highest potential to inhibit mtDNA synthesis (Brinkman et al., 1998). Weak inhibitors of DNA pol ɣ such as Lamivudine (3TC) and Tenofovir (TFV) were regarded as safer options in combination therapy (Curran and Ribera, 2011, Rosso et al., 2008). A study conducted by Birkus et. al. in 2002 evaluated the mt toxicity of various NRTIs in human hepatoma (HepG2) cells. The study concluded that the ranking of mt toxicity of the NRTIs studied from most severe to least severe were as follows: ddC > ddI > d4T > AZT > TFV = 3TC = ABC (Birkus et al., 2002). Another study by the same authors also assessed the cytotoxicity of NRTIs in HepG2 cells, human skeletal muscle and myeloid cells; the study ranked toxicity as follows: ddC > AZT > d4T > TFV > 3TC (Cihlar et al., 2002).

Inconsistencies have been observed in studies both *in vivo* and *in vitro* correlating mtDNA levels with mt toxicity and associated clinical outcomes (Apostolova et al., 2011). Tenofovir is a relatively new introduction to HAART and is considered a safer alternative due to its weak inhibition of DNA pol ɣ. However, TFV has been shown to induce mt structural changes and mt dysfunction while maintaining mtDNA levels in rat kidneys, contributing to nephropathy (Kohler et al., 2009). It is clear that mt toxicity exerted by NRTIs goes beyond DNA pol ɣ inhibition. Alternative mechanisms to NRTI induced toxicity that have been proposed include depletion of ATP, mt biogenesis, depleted native nucleotide pools, transcription of mtDNA, mt membrane integrity and protein synthesis (Setzer et al., 2008, Cohen, 2010).

2.3.2.3. Mitochondrial biogenesis

Respiratory capacity is dependent on the number of functional mitochondria. Mitochondrial biogenesis refers to the replication of mitochondria by division of existing mitochondria. The biogenesis of mitochondria is regulated transcriptionally by both the nuclear and mt genome. The main regulator of mt biogenesis is a nuclear encoded transcriptional co-activator: peroxisome

proliferator-activated receptor gamma co-activator 1 alpha (PGC-1α) (Wu et al., 1999). The activity of PGC-1α is induced as a metabolic adaptation to stress conditions or changes in energy status (Cantó and Auwerx, 2009). The role of PGC-1α in mt biogenesis is via its interaction with nuclear respiratory factor 1 and 2 (NRF1; NRF2); as well as up-regulating genes coding for ETC subunits – cytochrome c oxidase I and II and ATP synthase (Wu et al., 1999).

Together, PGC-1α and NRF1 coordinate the transcription of nuclear encoded genes required for mt biogenesis, most importantly activating mt transcription factor A (TFAM) (Gleyzer et al., 2005). This transcription factor is a high mobility group and DNA-binding protein. Upon induction by NRF1, TFAM translocates to the mitochondria where it binds to mtDNA, allowing transcription of the mitochondrial genome by DNA pol ɣ (Ngo et al., 2011). Furthermore, TFAM plays a role in the processing and packaging of mtDNA (Kaufman et al., 2007).

Mitochondrial biogenesis markers (PGC-1α; NRF1; TFAM) are known to be increased in the event of mt dysfunction and oxidative stress (Lee and Wei, 2005). This is likely to be a compensatory mechanism during mt dysfunction. Furthermore, PGC-1α also induces the expression of uncoupling protein 2 (UCP2) and superoxide dismutase (SOD) in defence against increased oxidative stress (Valle et al., 2005); thus PGC-1α acts as a stress response protein.

Studies on these parameters related to NRTI use are conflicting. Analysis of adipose tissue from HIV positive patients following six weeks of AZT/3TC/d4T triple therapy in an Australian cohort showed elevated expression of *NRF1*, *TFAM* and *PGC-1α* (Mallon et al., 2005). A similar study in the United Kingdom quantified gene expression in adipose tissue after 18-24 months of NRTI therapy. The results of the study showed TFV and AZT elevated *PGC-1α* expression, but reduced *NRF1* (Boothby et al., 2009). A South African cohort displayed a reduction in *NRF1* expression in adipocytes following 4 weeks treatment with d4T/EFV dual therapy (Menezes et al., 2013). Stavudine treated HepG2 cells, however, showed an increase in nuclear mt biogenesis transcripts (Setzer et al., 2008). From the above described literature it is clear that mt biogenesis genes are differentially expressed depending on tissue type and exposure time to NRTIs.

2.3.2.4. Mitochondrial free radical production

One of the most common outcomes of mt dysfunction is elevated mt free radical production. Naturally, the mt toxicity displayed by NRTIs is likely to contribute to an imbalance in cellular redox state. Previous studies have shown that a link exists between HAART use and elevated free radical production and markers for oxidative damage (Mondal et al., 2004, Ngondi et al., 2006, Masia et al., 2007).

Reactive oxygen species are a natural by-product of cellular respiration, making mitochondria the major endogenous source of ROS. Four electron transporting complexes (I-IV) and ATP synthase

facilitate OXPHOS, driving ATP synthesis (Figure 2.7). Products from the tricarboxylic acid (TCA) cycle, amino acid metabolism and β-oxidation all feed into ATP synthesis. An electron donor, usually NADH or $FADH_2$, is transferred through consecutive complexes via a series of redox reactions. Electron transfer is coupled with the transfer of protons (H^+) across the mt membrane, creating an electro-chemical gradient. The gradient across the inner mt membrane shunts ADP through ATP synthase, producing ATP. The final electron acceptor is molecular oxygen (O_2). Oxygen is then reduced to H_2O.

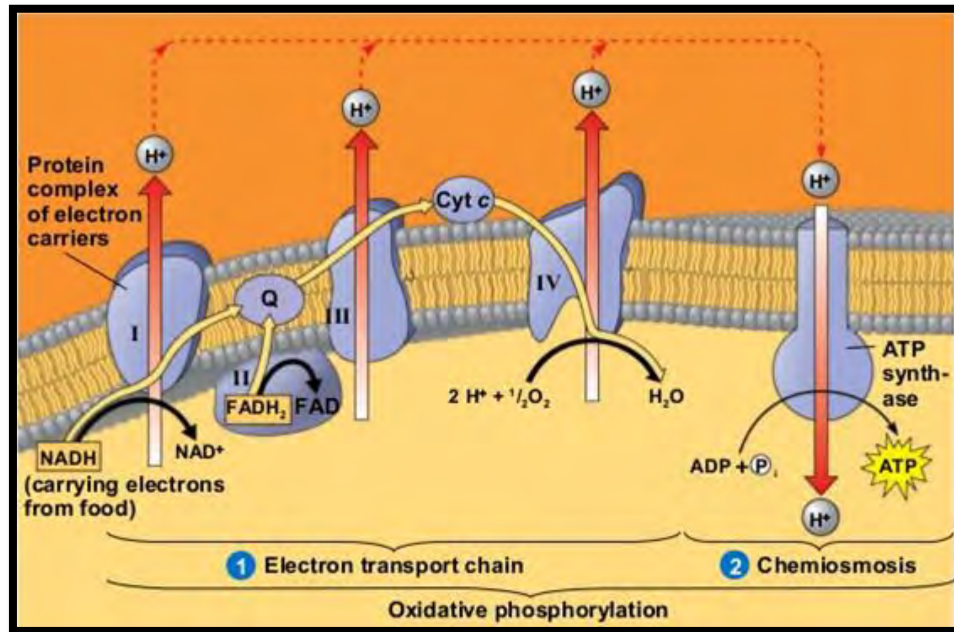

Figure 2.7: The electron transport chain is the site of oxidative phosphorylation that drives ATP synthesis. Complex I and III have been identified as sites of superoxide production due to electron leakage (Senft and Ronai, 2015)

Under normal physiological conditions, less than 1% of O_2 generated during ATP synthesis is converted to superoxide ($O_2^{\bullet-}$), a potent free radical (Raha and Robinson, 2000). Although an efficient means of energy production, the ETC is termed as "leaky" due to the escape of electrons during OXPHOS. Complex I and complex III have been identified as the main sites of electron leakage (Du et al., 1998, Beyer, 1992).

Basal levels of ROS have physiological relevance in cell signalling and immune response (Griendling et al., 2000, Thannickal and Fanburg, 2000). Insult to mitochondria, however, disrupts ETC function, changing the electro-chemical gradient across the inner mitochondrial membrane. This change elevates electron leakage from the ETC, thus increasing $O_2^{\bullet-}$ production. In the event that the cell cannot compensate for the excessive production of ROS, a phenomenon known as oxidative stress occurs.

2.3.2.5. Oxidative stress

Reactive oxygen species	Half-life
	s
$HO^{\bullet}$, hydroxyl radical	10^{-9}
$RO^{\bullet}$, alkoxyl radical	10^{-6}
$ROO^{\bullet}$, peroxyl radical	7
H_2O_2, hydrogen peroxide	-(enzymic)
$O_2^{\bullet -}$, superoxide anion radical	-(enzymic)
$^1O_2$, singlet oxygen	10^{-5}
$Q^{\bullet -}$, semiquinone radical	days
$NO^{\bullet}$, nitric oxide radical	1−10
$ONOO^-$, peroxynitrite	0.05−1

Figure 2.8: The most physiologically abundant reactive oxygen species (Sies, 1994)

Oxidative stress is an imbalance in the pro-oxidant/antioxidant system, in favour of the former, that often leads to pathological conditions and disease. Free radicals, including ROS, are molecules with free valence electrons. These molecules are extremely reactive as they are capable of "stealing" electrons from cellular macromolecules. Oxidation of cellular macromolecules including lipids, protein and DNA, results in detrimental changes to structure and function. At basal levels, ROS are required for intracellular redox signalling (Thannickal and Fanburg, 2000). An over-production of ROS, however, increases the rate of free radical reaction with cellular components. Processes that may lead to the excessive production of ROS include ionizing radiation, xenobiotic metabolism, enzymatic synthesis of nitric oxide, or oxidative burst in phagocytes (Bohr, 2002).

2.3.2.6. Oxidative DNA damage

The interaction of ROS with DNA bases results in the formation of adducts or lesions. Various adducts have been identified (Figure 2.9), however the most widely studied is the guanine 8-oxo-7,8-dihydroguanine (8-oxoG) (Fortini et al., 2003). This lesion is highly mutagenic and may contribute to carcinogenesis. The presence of the 8-oxoG adduct causes mispairing, leading to adenine to cytosine transversions during DNA replication, contributing to mutagenesis.

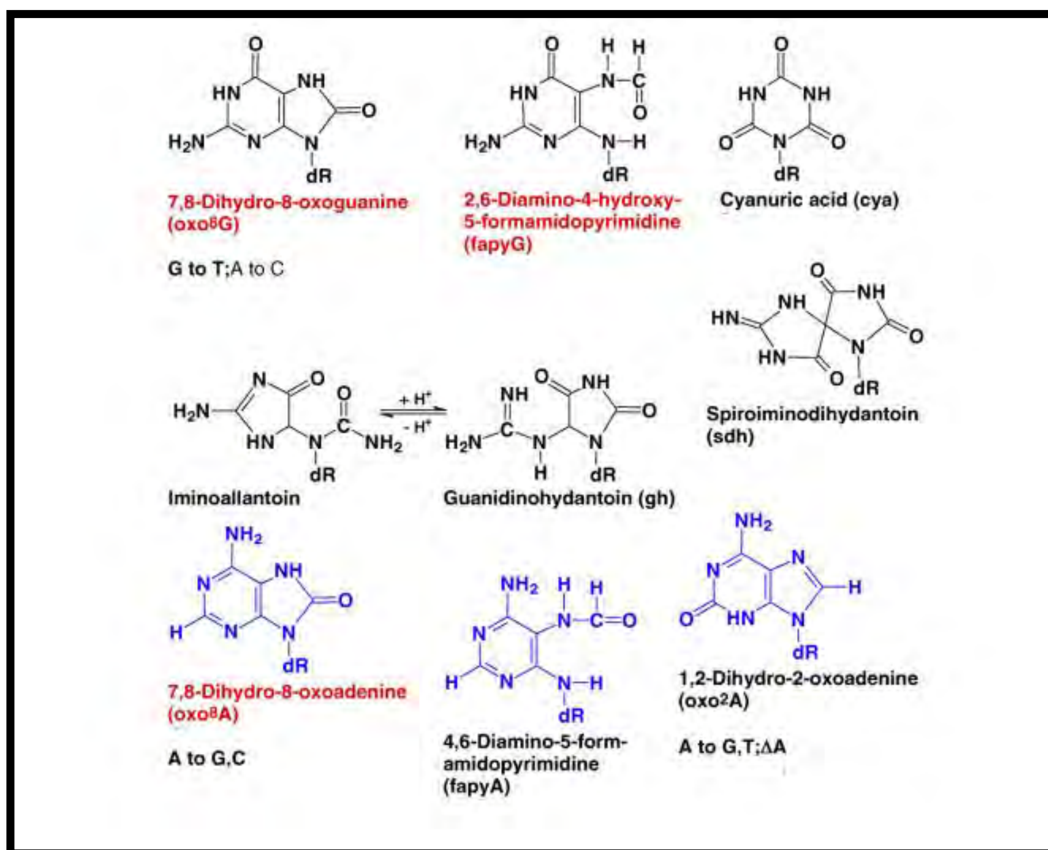

Figure 2.9: Oxidized guanine (black) and adenosine (blue) adducts. Substrates for OGG1 are indicated in red (Klungland and Bjelland, 2007)

Removal of the adduct is achieved in both nuclear DNA and mtDNA by the base excision repair enzyme, 8-oxoG DNA glycosylase (OGG1) (Stuart et al., 2004). This glycosylase reduces the accumulation of 8-oxoG DNA adducts by binding to DNA and cleaving the glycosidic bond of the DNA adduct. The activity of OGG1 in NRTI toxicity is particularly important as the repair activity of DNA pol ɣ is compromised.

2.3.2.7. Lipid peroxidation

Although proteins and DNA appear to be more significant targets of ROS, lipid peroxidation is a late stage effect of oxidative stress. Biological constituents that are high in polyunsaturated fatty acids (phospholipids, and glycolipids) and cholesterol are prime targets for oxidative degradation. Hydroxyl radicals (OH•) and hydroperoxyl radicals (OH_2) have a high affinity for the double bonds of the methylene bridges in polyunsaturated fatty acids. Initiation of lipid peroxidation occurs when a free radical extracts allylic hydrogen to form a carbon centred lipid radical. This "electron stealing" mechanism is then propagated once the lipid radical reacts with O_2, to form a peroxyl lipid radical. The peroxyl radical then "steals" a hydrogen atom from adjacent fatty acids, causing a chain reaction of lipid radical formation. This chain reaction is only terminated when two lipid radicals react to form

a non-radical product or through interaction with an antioxidant electron donor (Ayala et al., 2014, Kaufman et al., 2003). The products of lipid peroxidation are toxic and contribute to a variety of pathogenic conditions including asthma, neurodegenerative disorders, renal damage, pre-eclampsia and metabolic complications (Pham-Huy et al., 2008). The reactive aldehydes: malondialdehyde (MDA) and 4-hydroxynonenal are the most commonly studied products of lipid peroxidation and are measured as markers of oxidative stress (Requena et al., 1996). Lipid peroxides are usually detoxified by vitamin E or endogenous antioxidant enzymes SOD, catalase (CAT), and glutathione peroxidase (GPx).

2.3.2.8. Oxidative protein damage

Proteins have unique three-dimensional structures that are vital to their biological functionality. Protein side chains and polypeptide backbones of proteins are susceptible to oxidation by ROS, causing changes to the native protein structure. Oxidation of protein side chain residues cause partial unfolding of quaternary structure, while oxidation of protein backbones result in fragmentation (Stadtman and Berlett, 1998). The structural changes induced by ROS eventually lead to loss of function and contribute to pathological conditions.

Protein aggregation occurs when hydrophobic stretches of unfolded proteins are exposed following oxidative damage. This allows irregular protein interactions and covalent cross-links between proteins. There are two fates of protein aggregates:- enzymatic repair or proteolytic degradation. Large clusters of protein aggregates, however, may be difficult to remove proteolytically. The accumulation of protein aggregates poses a toxic threat, causing structural changes and hindering normal physiological processes (Squier, 2001). The inability to remove oxidized protein aggregates is believed to be a contributor to biological aging and age-associated degenerative diseases (Squier, 2001, Berlett and Stadtman, 1997).

Considering the array of physiological processes mediated by mitochondria, mt protein quality control is imperative to well-being. Several adverse health outcomes caused by long term NRTI use have been defined as age-associated disorders (Smith et al., 2012). Accelerated aging due to compromised mt protein quality control may be a molecular mechanism of toxicity in chronic NRTI use.

2.4. MITOCHONDRIAL PROTEIN QUALITY SURVEILLANCE

Protein quality control is an evolutionarily conserved mechanism to cope with stressors that cause protein misfolding and aggregation. In order to cope with ROS produced by the ETC, the mitochondrion possesses an intricate protein maintenance network. The network consists of protein chaperones, ATP-dependent proteases and regulatory molecules (Sauer et al., 2004, Bukau et al.,

2006). During stress conditions that disrupt protein homeostasis, a cellular stress response is induced to re-model protein folding and increase the activity of protein chaperones and proteases.

The ubiquitin-proteasome is generally responsible for the clearance of misfolded proteins or protein aggregates. Disruptions to mt protein homeostasis, however, cannot be reliant on cytosolic proteases as the mt double membrane hinders accessibility. Mitochondria possess endogenous proteases to process damaged proteins, and the products are shuttled by chaperones into the cytosol (Voos, 2009).

2.4.1. Lon Protease

Proteolytic clearance in the mt matrix is largely mediated by Lon protease (Lon), a member of the ATPases associated with diverse cellular activities (AAA+ family), encoded by *LONP1*. It is an ATP-dependent serine peptidase that is essential in mt protein quality control. Lon protease recognizes specific sequences that are usually located at the core of folded proteins (and thus inaccessible) but are exposed in unfolded proteins (Gur and Sauer, 2008). This enables Lon to distinguish between damaged and functional proteins in the mt matrix. Upon recognition of the target sequence, proteins are unfolded if necessary, and transferred through a pore to a degradation chamber (Figure 2.10). The AAA+ module, which is common to all AAA+ proteases, is a conserved ATP-binding site consisting of approximately 200 amino acids (Lee and Suzuki, 2008). Binding of ATP to the AAA+ module of Lon causes a conformational change, enabling Lon to become proteolytically active (Sauer et al., 2004).

An important substrate for the proteolytic activity of Lon in the mt matrix is oxidized aconitase (Bota and Davies, 2002). Aconitase, an enzyme regulating the TCA cycle, is sensitive to oxidative inactivation. Upon inactivation, mt aconitase begins to accumulate and aggregate in the mt matrix. The accumulation of oxidized mt aconitase is characteristic of biological aging (Yan et al., 1997). Proteolytic clearance of oxidized aconitase aggregates by Lon prevents detrimental effects on mt function and cell viability (Bota and Davies, 2002).

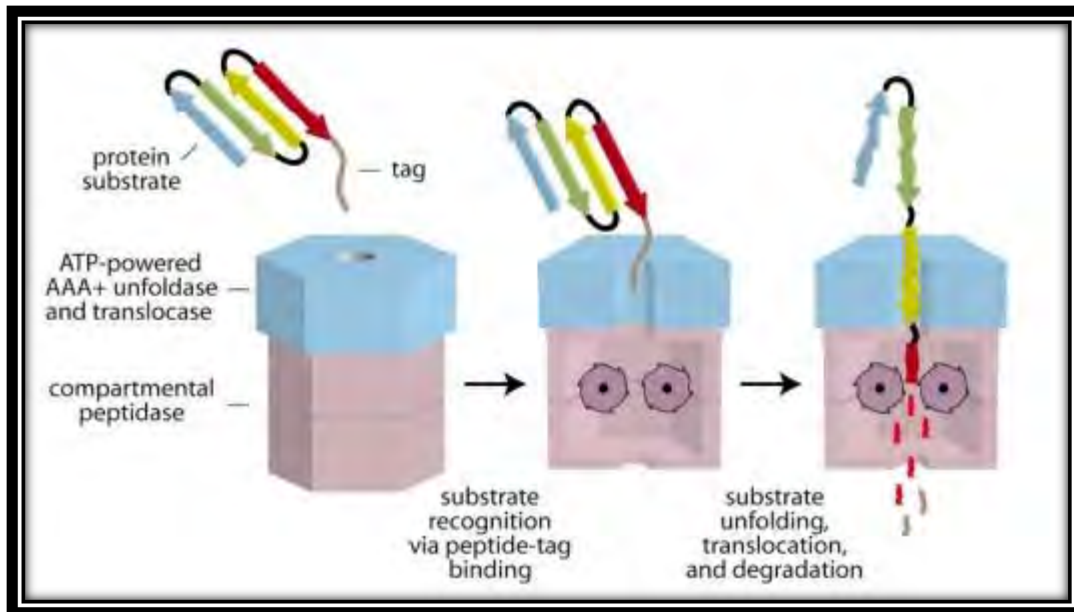

Figure 2.10: Schematic representation of mechanism of AAA+ protease activity. Recognition of unfolded/damages proteins by a peptide tag facilitates binding of the substrate to the protease. ATP-dependent unfolding enables the substrate to enter the degradation chamber (pink). Proteolysis occurs when ATP binds to the AAA+ module. Larger fragments may require ATPase activity to exit the chamber (Sauer et al., 2004)

Lon also plays a role in metabolic homeostasis due to its selective degradation of regulatory molecules in mt biogenesis. Post-translational phosphorylation of TFAM reduces the DNA-binding capability of TFAM, and marks the transcription factor for degradation by Lon (Lu et al., 2013). RNA interference of Lon increases both TFAM and mtDNA levels, validating the role of Lon in mt biogenesis (Matsushima et al., 2010). The chaperone activity of Lon is also crucial to the assembly of the mt subunits that form the ETC (Rep et al., 1996).

Due to the importance of Lon in mt protein maintenance, it has become a target for mt diseases and age-associated disorders. To date, only one study has related *Lon* mRNA expression to HAART and lipodystrophy (Pinti et al., 2010). The study showed significant elevation of *Lon* mRNA levels in adipose tissue obtained from patients on HAART presenting with lipodystrophy. Although this finding is highly relevant to NRTI toxicity, Lon protein levels were not evaluated as there is poor correlation between Lon mRNA and protein levels (Gibellini et al., 2014).

2.4.2. Post translational regulation of Lon – Sirtuin 3

The disjoint between Lon mRNA and protein levels may be due to the rapid response requirement of stress proteins. Hypoxia, disrupted energy production, oxidative stress and ER stress are known to induce Lon activity (Hori et al., 2002). Post-translation modifications provide a means to increase protein turnover without the time consuming synthesis, processing and exportation of *de novo* mRNA

synthesis. A recently identified regulator of Lon is a homolog of the sirtuin (SIRT) family, SIRT3 (Gibellini et al., 2014).

Sirtuins are NAD^+ dependent deacetylases that regulate various biological processes including cell death, metabolism, genomic stability, aging, and antioxidant response (Finkel et al., 2009). Seven homologs of this evolutionarily conserved family of proteins exist in mammals i.e. SIRT1-7. Substrates and cellular localization may differ between the various SIRTs. Sirtuin 1 is the most extensively studied, and along with SIRT 6 and 7, resides in the nucleus. Sirtuin 2 has been identified as a cytoplasmic SIRT. Mitochondrial located SIRTs include SIRT3-5 (Haigis and Guarente, 2006). The mt SIRTs are key regulators in cellular metabolism. This has made SIRTs a promising target in diabetes and metabolic syndrome (Guarente, 2006, Jiang, 2008). Due to its integral role in the mitochondria, the role of SIRT3 in mt maintenance will be described.

Sirtuin 3 is vital to mt function as it regulates mt antioxidant response, mt biogenesis (Kong et al., 2010, Brenmoehl and Hoeflich, 2013) and recently reported, mt unfolded protein response (UPR^{mt}) (Papa and Germain, 2014). The UPR^{mt} is a homeostatic response to maintain the protein folding environment in the mitochondrion, as well as mt architecture. Traditionally, UPR^{mt} is thought to be mediated by CCAAT-enhancer-binding homologous protein (CHOP) and oestrogen receptor α. Sirtuin 3, however, is rapidly emerging as regulator of UPR^{mt} through the induction of mt antioxidants SOD2 and PGC-1α, regulation of mitophagy (Papa and Germain, 2014) and post-translational modification of Lon (Gibellini et al., 2014). A recent study by Gibellini et al. (2014) demonstrated that not only does SIRT3 regulate acetylation (Lys917) status of Lon, but directly interacts with Lon, altering its expression. Acetylation is a well-established form of post-translation modification, with one third of mt proteins estimated to be acetylated (Zhao et al., 2010). Although the exact role of lysine acetylation has not been uncovered, it is suggested that Lon in its acetylated form is more stable.

2.4.3. Lon protease and endoplasmic reticulum stress

The ER is the hub of protein processing, folding and packaging and is essential to maintaining protein homeostasis. The mitochondrion and ER possess co-regulatory mechanisms during stress response. Transfer of lipids, metabolites, Ca^{2+} and peptides by chaperones facilitate the cross-talk between the ER and mitochondrion. Due to the inter-organelle communication, an insult to mt function elicits an ER response, and *vice versa* (Malhotra and Kaufman, 2011). The unfolded protein response (UPR) is part of the ER stress response and is an example of co-ordinated mt and ER stress (Senft and Ronai, 2015).

Endoplasmic reticulum stress is characterised as an accumulation of misfolded proteins in the lumen of the ER. Oxidative stress and mt dysfunction contribute to an elevation in misfolded proteins, often exacerbating ER stress (Xu et al., 2005, Haynes et al., 2004). The ER stress response is a cellular

response to restore homeostasis by two mechanisms: - 1) increasing the protein folding rate; 2) reducing the client protein load entering the ER. Reduction in the influx of client proteins is mediated by three main ER stress responses: protein kinase RNA-like ER kinase (PERK), activating transcription factor (ATF) 6, and inositol-requiring enzyme 1 (IRE1). The molecular mechanisms of these three sensors in the unfolded protein response (UPR) are described in Figure 2.11.

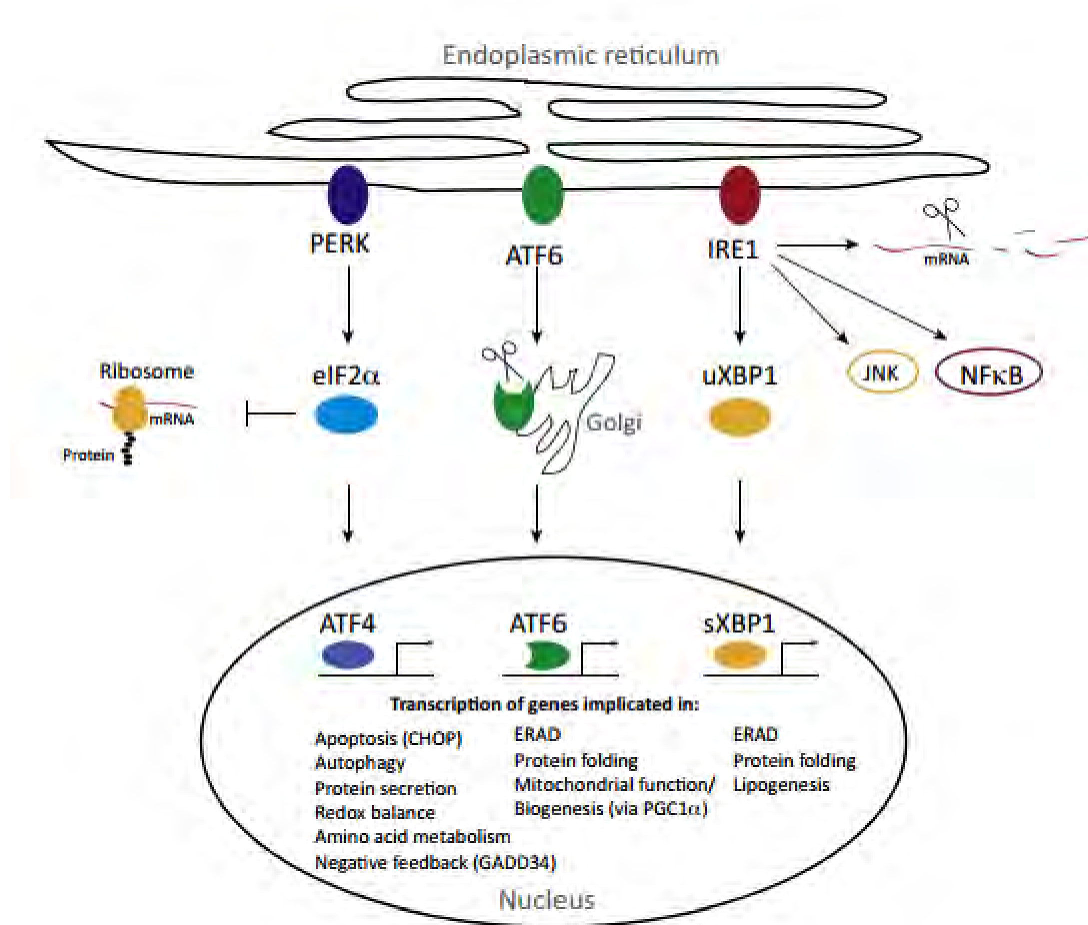

Figure 2.11: Molecular mechanisms of endoplasmic reticulum mediated unfolded protein response. The PERK mediated response is an effort to reduce translation by phosphorylation of eukaryotic translation initiation factor 2α (eIF2α). Activating transcription factor 6 (ATF6) accelerates folding and packaging of proteins in the Golgi apparatus. Inositol-requiring enzyme (IRE1) enhances degradation of mRNA and activates stress protein c-Jun N-terminal kinase (JNK) and inflammatory nuclear factor kappa-light chain enhancer of activated B cells (NFκB). The activation of JNK amplifies the activity of eiF2α to reduce protein influx (Senft and Ronai, 2015)

The chaperone activity of Lon has been identified as key role player in the transmission of ER stress to the mitochondrion (Hori et al., 2002). Silencing of PERK prevented the induction of Lon and the

recruitment of heat shock protein (HSP) 60 and mt HSP70 to form a complex with Lon. Heat shock proteins are ATP-dependent pro-survival molecules that are ubiquitously expressed. Heat shock protein 60 is localized in the mitochondria and regulates protein folding and chaperoning in the mt matrix, as well as mtDNA assembly (Kaufman et al., 2003). The complex formed with Lon, HSP60 and mtHSP70 is required for mt mediated cell death and protein assembly (Kao et al., 2015, Bender et al., 2011). The regulatory role of ER stress response proteins on the assembly of this complex provides a mt response to ER stress.

2.5. ENDOGENOUS ANTIOXIDANT DEFENCE MECHANISMS

The chemical modification of xenobiotics is an adaptive response to aid in the excretion of drugs. Phase I enzymes in biotransformation largely consist of CYP450 mono-oxygenases, which often produce electrophilic metabolites (Payne et al., 1987). Substrates of CYP450 mono-oxygenases include AZT and 3TC (Veal and Back, 1995), to a lesser extent d4T (Kaul et al., 1999), but not TFV (Chittick et al., 2006). The production of CYP450 metabolites stimulate phase II enzymes, which facilitate the conjugation of the electrophiles to reduced glutathione (GSH) and glucuronic acid. The liver is central to xenobiotic metabolism due to the high expression of both phase I and II enzymes. Therefore the liver bears the brunt of electrophilic attack caused by xenobiotic metabolism (Park et al., 1995). Under these circumstances, intricately co-ordinated cellular defence mechanisms are in place to maintain redox status and contain oxidative damage. The endogenous antioxidant defence system is an indispensable component to the cytoprotective armoury. Antioxidants scavenge free radicals by acting as electron donors, undergoing oxidation themselves. In order for this reaction to occur antioxidants require reducing capabilities, e.g. thiols, polyphenols.

2.5.1. Superoxide detoxification

The $O_2^{\bullet-}$ anion radical is one of the most abundantly produced ROS from the ETC. The products of $O_2^{\bullet-}$ reactions often yield other reactive species, perpetuating oxidative insult (Sies, 1991). The first line of defence against $O_2^{\bullet-}$ is SOD. Superoxide dismutase is one of the most extensively studied enzymatic antioxidants. The enzyme exists in different isoforms depending on its sub-cellular location: SOD1 (cytosol; mitochondrion), SOD2 (mitochondrion), and SOD3 (extracellular) (McCord and Fridovich, 1988, Tainer et al., 1983). Superoxide dismutase converts $O_2^{\bullet-}$ to hydrogen peroxide (H_2O_2). Hydrogen peroxide may be rapidly converted to OH• via the Fenton reaction, and thus requires further processing by CAT to form H_2O (Figure 2.12).

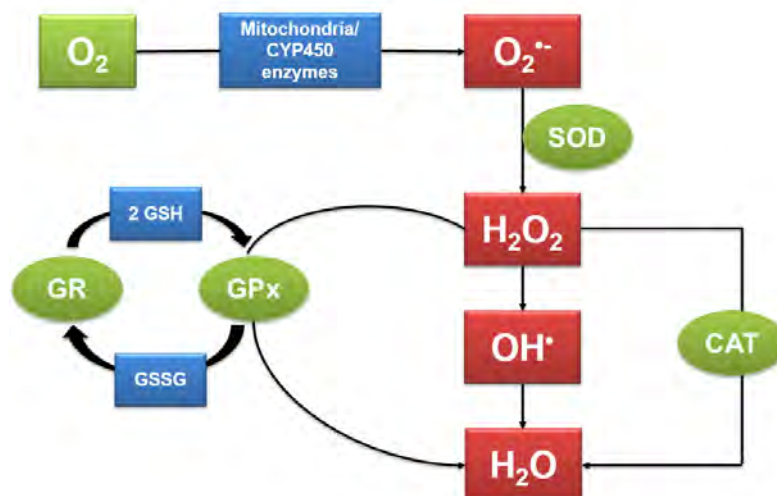

Figure 2.12: Schematic representation of the first line of defence against mitochondrial ROS production. Superoxide dismutase (SOD) catalyzes the reaction of $O_2^{\bullet-}$ to H_2O_2. Hydrogen peroxide may be further processed by catalase (CAT) or glutathione peroxidase (GPx). Glutathione peroxidase mediates the donation of electrons from reduced glutathione (GSH) to convert H_2O_2 to H_2O. Oxidized GSH (GSSG) may be converted back to the reduced state by glutathione reductase –GR (Figure by author)

2.5.2. Glutathione antioxidant defence

The GSH mediated antioxidant response is one of the most important phase II detoxification pathways in drug metabolism (Figure 2.12). The thiol groups present in GSH give this antioxidant its biological activity. The sulfhydryl groups act as proton donors, converting disulphide bonds in oxidized proteins to cysteine. Two GSH molecules may also react with H_2O_2 to yield water and oxidized GSH (GSSG) in a reaction catalysed by GPx (Wendel, 1980). Reduced glutathione may be regenerated by acceptance of an electron from NADPH. The regeneration of GSH is mediated by the activity of glutathione reductase (GR). Depletion of GSH is often regarded as an indicator of cytotoxicity (Arrick and Nathan, 1984). Selenium is necessary for GPx activity, thus its depletion compromises the antioxidant response (Rotruck et al., 1973).

2.5.3. Master regulator of antioxidant response - Nuclear factor erythroid 2-related factor 2 (Nrf2)

Stress responses, including antioxidant defence, require rapid response and tightly regulated systems. The antioxidant response element (ARE) is a cis-acting element that is located either on the promoter or upstream of the promoters of major phase II and antioxidant genes (Kobayashi and Yamamoto, 2006). Transcriptional induction of the ARE allows for the expression of a battery of cytoprotective genes including GPx, thioredoxin, SOD, heme-oxygenase 1 (HO-1), peroxidases and glutathione-S-transferase. Since the identification of the ARE, the role of the nuclear-erythroid 2-related factor 2

(Nrf2)/ Kelch-like erythroid cell derived protein with CNC homology (ECH)-associated protein (Keap1) pathway in cellular defence has become a focus in antioxidant research.

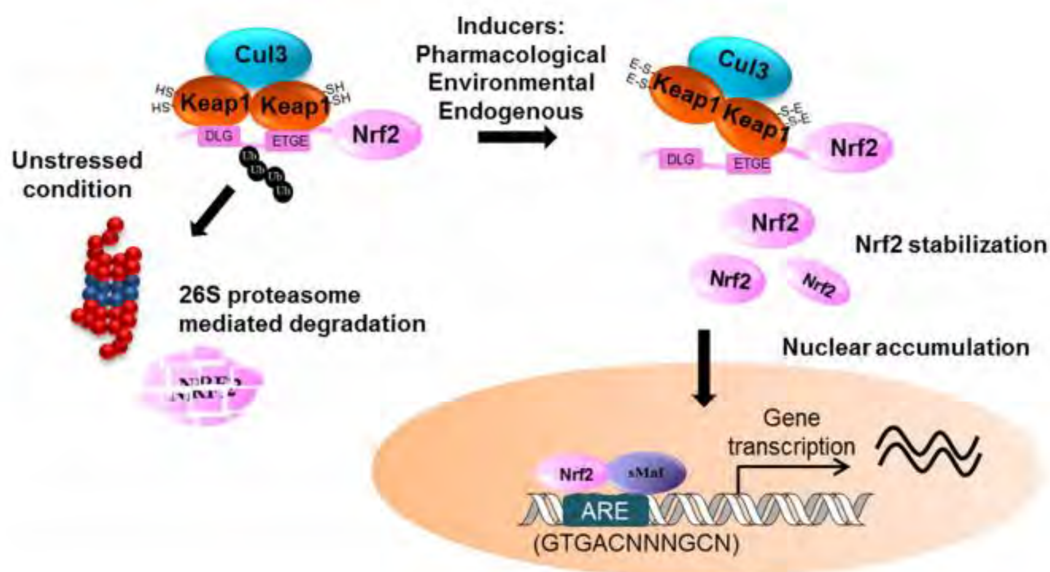

Figure 2.13: The initiation of antioxidant stress response by Nrf2. Upon oxidation of cysteine residues binding the Nrf2/Keap1 complex, Nrf2 translocates to the nucleus and forms a homodimer with a small Maf protein. Following recognition of a consensus sequence, the Nrf2/Maf complex binds to the ARE and induces the transcription of antioxidant defence and transporter genes. Under normal conditions, Nrf2 is marked for degradation by the ubiquitin proteasome (Huang et al., 2015)

Nuclear-erythroid 2-related factor 2 is a basic region leucine zipper (bZIP) transcription factor that belongs to the Cap ‘n’ Collar (CNC) family (Moi et al., 1994). Tissue involved in detoxification (liver, kidney) and organs with exposure to the external environment (lungs, skin, gastrointestinal tract) have elevated expression of Nrf2 (Copple et al., 2008). The mechanism by which Nrf2 induces transcription of the ARE is shown in Figure 2.13. Under normal conditions, Nrf2 is bound to its inhibitor Keap1 in the cytoplasm (Itoh et al., 1997) and is marked for degradation by the ubiquitin proteasome. An increase in electrophiles, however, causes oxidation of the cysteine residues that tether Nrf2 to Keap1 (Stewart et al., 2003). The free Nrf2 undergoes phosphorylation at the transactivation domain, and subsequently translocates to the nucleus. At the nucleus, Nrf2 recognizes the ARE and through heterodimerisation with small Maf proteins, binds to the ARE, initiating transcription of antioxidant genes (Motohashi and Yamamoto, 2004).

The importance of Nrf2 in protection against drug induced cytotoxicity has been well established (Copple et al., 2008). Although the classical implication of Nrf2 in cellular defence is related to antioxidant induction, alternative roles of the transcription factor are quickly emerging. The binding

of Nrf2 to the ARE is mediated by recognition of a consensus sequence (Figure 2.13). The ARE, however, shares motifs with the mt biogenesis regulator, NRF1. Due to these shared motifs, Nrf2 has been shown to interact with NRF1, mediating mt biogenesis (Piantadosi et al., 2008). A role for Nrf2 in metabolic reprogramming has also been identified (Mitsuishi et al., 2012). An interesting concept that has yet to gain momentum is the role of Nrf2 in xenobiotic detoxification via induction of drug transporters (Aleksunes et al., 2008, Maher et al., 2005). The diverse protective activity of Nrf2 is an important cytoprotective response in drug-induced toxicity.

2.6. DETOXIFICATION BY ATP-DEPENDENT EFFLUX DRUGS TRANSPORTERS

Drug metabolizing enzymes and transporters are determinants of drug efficacy, toxicity and excretion. Charged or polar drugs are unable to passively cross cell membranes. (Doring and Petzinger, 2014). Two major drug transporter families are important: solute carrier proteins (influx) and ATP-binding cassette (*ABC*) proteins (efflux). The role of these transporters in drug metabolism and processing is regarded as phase 0 and phase III respectively. Phase III proteins aid in reducing the risk of bioaccumulation of xenobiotics.

2.6.1. ATP-binding cassette family of transporters

The *ABC* super-family of transporters are energy dependent membrane proteins that actively transport both endogenous and pharmacological agents, including anti-cancer and anti-viral drugs. The *ABC* proteins have a commonality in structure, consisting of a transmembrane domain (TMD) and nucleotide binding domain (NBD). The TMD spans the membrane and varies among subtypes of *ABC* proteins. This domain is responsible for the recognition of substrates and undergoes a conformational change to facilitate transport of the substrate across the cell membrane. The NBD, which is essentially the "ATP-binding cassette", contains a catalytic core and an α-helical subdomain (Vasiliou et al., 2009). Hydrolysis of ATP in the catalytic core enables the TMD to undergo conformational change. The 49 *ABC* genes that have been identified are subcategorized into seven families (*ABCA; ABCB; ABCC; ABCCD; ABCCE; ABCCF; ABCCG*). Each family codes for protein transporters known under different names. The *ABCC* class of transporters code for multi-drug resistance proteins (MRPs); this transporter was first recognized for conferring resistance to chemotherapeutic agents (Yokoyama et al., 1999, Chan et al., 1997, Stavrovskaya and Stromskaya, 2008).

2.6.2. Multi-drug resistance proteins

There are twelve classes of MRPs (MRP1-MRP12), all coded for by respective *ABCC* genes. Multi-drug resistance proteins mediate energy dependent efflux of xenobiotic compounds, lowering the intracellular concentration. The substrate range of MRPs is broad, including prostaglandins (Reid et al., 2003), bile salts and conjugates, steroids (Zelcer et al., 2003), glutathione conjugates (Rius et al.,

2003), cyclic nucleotides (Wielinga et al., 2003), antiviral drugs:- TFV and Adefovir (Imaoka et al., 2007), antihypertensive drugs:- furosemide and hydrochlorothiazide (Hasegawa et al., 2007), and anticancer drugs:- camptothecin (Tian et al., 2005), methotrexate (Chen et al., 2002), and topotecan (Tian et al., 2006). The major MRPs involved in detoxification are those expressed in the liver and kidney. These include MRP2, MRP4 and MRP5 (Keppler, 2011, Ritter et al., 2005).The unique ability of MRP4 (coded for by *ABCC4*) to transport cyclic nucleotides, antiviral drugs and their metabolites is of relevance to NRTI clearance from hepatocytes.

2.6.3. Multi-drug resistance protein 4 (ABCC4)

Multi-drug resistance protein 4 is expressed in renal tubules, capillaries in the brain, blood cells and the basolateral membrane of hepatocytes (Ritter et al., 2005). The protein was first reported in T-lymphoid cells and was associated with resistance to nucleoside analogues used in HAART, including AZT and d4T (Schuetz et al., 1999). The protein quickly gained notoriety for drug resistance, however, the ability of MRP4 to transport nucleotides, conjugated steroids and ADP showed MRP4 was also involved in cell signalling (Sellers et al., 2012, Sinha et al., 2015, Russel et al., 2008). The high expression of *ABCC4*/MRP4 in the liver and kidney has also led to the assumption MRP4 may reduce the risk of xenobiotic bioaccumulation at drug deposition sites. This idea is reiterated by the induction of MRP4 by stress response transcription factor, Nrf2 (Maher et al., 2008, Satoh et al., 2013).

Expression of MRP4/*ABCC4* is variable, with poor correlations between mRNA and protein levels (Markova and Kroetz, 2014). The *ABCC4* gene is highly polymorphic and studies have evaluated the effect of different single nucleotide polymorphisms on the expression and activity of MRP4 (Abla et al., 2008, Kelly et al., 2010, Markova and Kroetz, 2014). Differences in the DNA sequence of the *ABCC4* promoter, however, do not entirely account for the regulation of gene expression. A recent study by Markova and Kroetz (2014) reported a novel epigenetic mechanism of *ABCC4* modulation by microRNA (miR-) 124a and miR-501 (Markova and Kroetz, 2014).

2.6.4. MicroRNA and drug transporters

MicroRNA (miRNAs) are an abundant class of short non-coding RNA that fall under the field of epigenetics. Epigenetics refers to genomic modifications independent of changes to the actual gene sequence. Examples of epigenetic modifications include DNA methylation, chromatin and histone modification and miRNAs. MicroRNAs are 18-25 nucleotides long and play a role in post-translational silencing. The full description of miRNA biogenesis and mode of action are described in Figure 2.14.

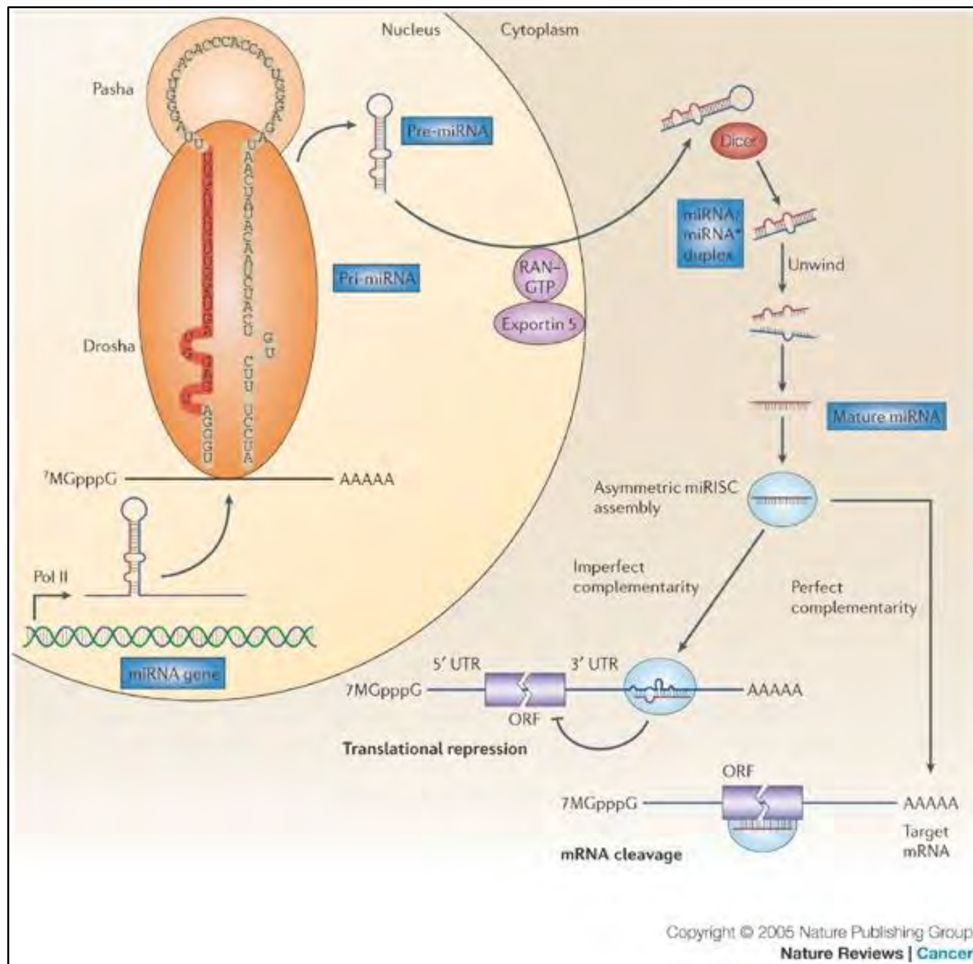

Figure 2.14: Mechanism of microRNA (miRNA) biogenesis and mode of RNA silencing. MicroRNAs can be generated from either their own genes or the introns of protein-coding genes and are transcribed as independent units. Transcription of miRNA is carried out by RNA polymerase II (Pol II), producing large capped primary transcripts (Pri-miRNA). Primary transcripts are characterized by stem loops. The RNA III enzyme, Drosha, together with its co-factor (Pasha) cleave pri-miRNA at the stem of the loop, releasing 70-nucleotide long pre-miRNA products. The nuclear transporter, Exportin 5, and GTPase Ran facilitate the export of the pre-miRNA from the nucleus to the cytoplasm. In the cytoplasm, pre-miRNA is subject to further processing by Dicer, producing a miRNA duplex. The duplex is loaded into an RNA-inducing silencing complex (RISC), consisting of multiple proteins, including Argonaute proteins. Mature miRNA are unwound and remain tethered to Argonaute proteins within the complex. MicroRNA have more than one target gene. The mature miRNA binds the 3' untranslated region (UTR) of the target gene, under the guidance of Argonaute protein, by complementary base pairing. Perfect complementarity with a target gene results in degradation of the target mRNA. Imperfect complementarity causes translational repression (Esquela-Kerscher and Slack, 2006)

MicroRNA have been implicated in various biological processes and pathological conditions including cancer (Jansson and Lund, 2012), diabetes (McClelland and Kantharidis, 2014), cardiovascular disease (Small et al., 2010) and recently drug transport and metabolism (Peng and Zhong, 2015). The regulatory role of miRNAs on *ABC* transporters has been confirmed in hepatocellular carcinoma. Thirteen miRNAs are associated with the modulation of gene expression of *ABCA1*, *ABCC1*, *ABCC5*, *ABCC10*, and *ABCE1* (Borel et al., 2012). The transporter *ABCC1* is regulated by miR-1291 in pancreatic cancer (Pan et al., 2013) and miR-134 in breast cancer cells (Lu et al., 2015). Multi-drug resistance protein 1 (coded for by *ABCC1*) was also found to be negatively regulated by miR-27a and miR-451 (Zhu et al., 2008). The majority of these studies have focussed on cancer and chemotherapeutic agents. The impact of miRNA regulation in determining drug toxicity through cellular transporters will have significant implications for long term therapies such as HAART.

The adverse health outcomes of long term NRTI (lipodystrophy, metabolic syndrome, cardiomyopathy, neuropathy, renal dysfunction) can all be related to mt dysfunction. Although mt toxicity has been established as a mechanism of NRTI toxicity, there are still gaps in the knowledge of molecular events that lead to adverse clinical outcomes. The effects of NRTI use on redox status, antioxidant response, mitochondrial biogenesis and cellular detoxification mechanisms is still not clearly defined. Furthermore, the epigenetic modulation of these processes in relation to NRTI toxicity is also not widely known. Investigations into the biochemical outcomes of acute and prolonged exposure at plasma level concentrations will provide physiologically relevant insight and enable targeted therapeutic intervention.

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CHAPTER THREE

Mitochondrial and oxidative stress response in HepG2 cells following acute and prolonged exposure to antiretroviral drugs

Savania Nagiah[a], Dr Alisa Phulukdaree[a1], Prof Anil Chuturgoon[a]

[a] Discipline of Medical Biochemistry, School of Laboratory Medicine and Medical Sciences, University of KwaZulu-Natal, Durban, South Africa

[1] Present Address: Department of Physiology, Faculty of Health Sciences, Prinshof Campus, University of Pretoria, Pretoria, South Africa

Address all correspondence to Prof Anil Chuturgoon, Discipline of Medical Biochemistry and Chemical Pathology, Faculty of Health Sciences, George Campbell Building, Howard College, University of KwaZulu Natal, Durban, 4041, South Africa. Telephone: (031) 260 4404. Email: chutur@ukzn.ac.za

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- **PGC-1α**
- **Nuclear-erythroid2 related factor 2**
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Abstract

Chronic HIV treatment with antiretroviral drugs has been associated with adverse health outcomes. Mitochondrial toxicity exhibited by nucleoside reverse transcriptase inhibitors (NRTIs) is pinpointed as a molecular mechanism of toxicity.

This study evaluated the effect of NRTIs: Zidovudine (AZT, 7.1µM), Stavudine (d4T, 4µM) and Tenofovir (TFV, 1.2µM), on mitochondrial (mt) stress response, mtDNA integrity and oxidative stress response in human hepatoma cells at 24h and 120h. Markers for mt function, mt biogenesis, oxidative stress parameters, and antioxidant response were evaluated by spectrophotometry, luminometry, flow cytometry, qPCR and western blots.

We found that AZT and d4T reduced mtDNA integrity (120h, AZT: 76.1%; d4T:36.1%, $p<0.05$) and remained unchanged with TFV. All three NRTIs, however, reduced ATP levels (AZT: 38%; d4T: 56.4%; TFV: 27.4%-, $p=0.01$) and mt membrane potential at 120h ($p<0.005$). Oxidative damage and reactive oxygen species were increased by TFV and AZT at 24h, and by d4T at 120h ($p<0.05$). Antioxidant response molecules and mt biogenesis markers were elevated by all NRTIs, with TFV causing the most significant increase ($p<0.05$).

Data from this study suggests that AZT, d4T and TFV alter mt function. TFV, however, achieves this independently of mtDNA depletion. Furthermore, AZT exerts toxicity soon after exposure as noted from changes at 24h and d4T exerts greater toxicity over prolonged exposure (120h).

Introduction

Nucleoside reverse transcriptase inhibitors (NRTIs) form the backbone of combination antiretroviral therapy in HIV treatment. In developing countries, the massive rollout of these drugs favour standardized fixed dose combinations due to limited resources and cost effectiveness. Under these circumstances, the long term use of these drugs has seen the emergence of adverse health outcomes, particularly changes to metabolic parameters, lipodystrophy, dyslipidemia and hepatic steatosis [Carr and Cooper, 2000]. Investigation into antiretroviral therapy and their associated disorders found individual NRTIs had varying degrees of toxicity that are tissue specific and time dependent [Birkus et al., 2002; Bleeker-Rovers et al., 2000; Igoudjil et al., 2007; Kline et al., 2009].

Mitochondrial (mt) toxicity is a common outcome of NRTI use. Initially, thymidine analogues, including Zidovudine (AZT) and Stavudine (d4T), were considered to be the main culprits due to the inhibitory effect of these drugs on mtDNA replication [Brinkman et al., 1998]. However, other NRTIs, such as Tenofovir (TFV), still exhibit mt toxicity in spite of maintaining mtDNA levels [Kohler et al., 2009; Lebrecht et al., 2009]. This opened up investigations into alternate mechanisms of mt toxicity. Other proposed targets of NRTI toxicity include ATP synthesis, mt biogenesis, depleted native nucleotide pools, transcription of mtDNA, mt membrane integrity and transport and protein synthesis [Cohen, 2010; Setzer et al., 2008].

Oxidative stress and mt dysfunction are two closely linked outcomes of xenobiotic metabolism. Disruptions to mt function increase the production of reactive oxygen species (ROS), especially superoxide ($O_2^{\bullet -}$), via defective oxidative phosphorylation. Elevated free radical production over a period of time depletes the antioxidant defence response, eventually resulting in oxidative damage to bio-macromolecules including DNA, protein and lipid membranes.

The antioxidant defence system is regulated by the transcription factor, nuclear factor erythroid 2-related factor 2 (Nrf2). Nrf2 exists in the cytoplasm, bound to its inhibitor, Kelch-like associated ECH-associated protein (Keap1). In this state, Nrf2 is marked for degradation by ubiquitin, maintaining Nrf2 at low basal levels. Elevated ROS levels cause dissociation of Nrf2 from Keap1 by oxidizing the cysteine residues of the Nrf2-Keap1 complex. This allows Nrf2 to translocate to the nucleus and bind to the antioxidant response element (ARE), initiating the transcription of antioxidant (AO) genes such as superoxide dismutase (SOD), glutathione peroxidase (GPx), glutathione reductase, and catalase [Motohashi and Yamamoto, 2004]. Aside from the endogenous antioxidant defence system, repair mechanisms are in place to compensate for oxidative damage. The high affinity for ROS to the guanine base in DNA results in DNA mutations and lesions. A base excision repair response to both nuclear and mtDNA damage is modulated by the enzyme, 8-oxoguanine DNA glycosylase (OGG1) [Stuart et al., 2004].

Mitochondria, being the main endogenous source of ROS, also require specialized defence mechanisms. An inner mitochondrial membrane protein, uncoupling protein2 (UCP2), is one such mechanism that functions to reduce ROS production by uncoupling oxidative phosphorylation from ATP synthesis. It achieves this by increasing proton conductance across the inner mt membrane, thus reducing ROS production during ATP synthesis [Nedergaard et al., 2005]. A common observation of mt toxicity is the up-regulation of mt biogenesis markers. The transcription of mt genes and mt biogenesis is regulated by a transcription co-activator, peroxisome proliferator-activated receptor γ co activator 1α (PGC-1α). PGC-1α regulates mt gene transcription via interaction with nuclear respiratory factor 1(NRF1) and 2, which in turn activates mt transcription factor A (TFAM) [Mallon et al., 2005]. Previous studies found that both PGC-1α and TFAM expression was elevated in the presence of NRTIs, suggesting a nuclear response to mt toxicity [Mallon et al., 2005; Setzer et al., 2008]. Aside from its role in mt biogenesis, PGC-1α also acts as a modulator of mt AO response by increasing expression of oxidative stress protective genes [St-Pierre et al., 2006; Valle et al., 2005]. Mitochondrial AO responses such as PGC-1α and UCP2 are of importance as a known outcome of NRTI toxicity is oxidative stress [Lewis et al., 2001; Manda et al., 2011].

Naturally, the nature of NRTI toxicity results in mitochondria-rich tissues being the most affected following chronic exposure [Dagan et al., 2002]. The liver being the metabolic hub of humans is abundant in mitochondria. The HepG2 cell line has been widely used in previous studies evaluating the effect of antiretroviral drugs on mitochondrial toxicity [Birkus et al., 2002; de Baar et al., 2007; Setzer et al., 2008; Velsor et al., 2004; Walker et al., 2002]. HepG2 cells possess cytochrome P_{450} activity and has hence been identified as an early model for xenobiotic metabolism [Roe et al., 1993]. Nucleoside reverse transcriptase inhibitors, including AZT and d4T, require intracellular phosphorylation to their triphosphate active form. It is only in this form that the drugs may incorporate into DNA, exerting an effect. The cytochrome P_{450} activity of HepG2 cells is integral to the biotransformation of NRTIs. This study aimed to investigate the effects of three commonly used NRTIs: AZT, d4T and TFV on mt function and AO response; focussing on Nrf2 and PGC-1α mediated stress responses, in HepG2 liver cells following exposure for 24 hours (h) and 120 h.

Materials and Methods

Materials

All reagents were purchased from Merck (Darmstadt, Germany), unless otherwise stated. Cell culture media were obtained from Lonza, Biowhittaker (South Africa), and supplements were purchased from Sigma-Aldrich (St Louis, MO, USA). All antiretroviral drugs were obtained from the NIH Aids reagents programme. Antibodies utilized for western blots were purchased from Cell Signalling Technology, Inc (Massachusetts, USA).

Cell culture and treatment

Human hepatoma (HepG2) cells were cultured in supplemented Eagle's minimum essential media (10% foetal calf serum, 1% penstrepfungizone, 1% L-glutamine) at 37°C in a humidified incubator. Cells were seeded in sterile 25cm^3 cell culture flasks and subject to treatment once the cells were 80% confluent.

Stocks of NRTIs (10mM in dimethylsulphoxide) were prepared. HepG2 cells were treated with NRTIs at maximum plasma level concentrations [Venhoff et al., 2007; Walker et al., 2002]. Treatments with AZT (7.1µM), d4T (4µM), TFV (1.2µM) and an untreated control were conducted over two time periods (24 h; 120 h). For the 120 h treatment, fresh cell culture medium containing NRTI treatment was replenished every 48 h.

Lipid peroxidation

Lipid peroxidation was used as a marker of oxidative stress. The thiobarbituric reactive substances (TBARS) assay was used to quantify extracellular malondialdehyde (MDA), a by-product of lipid peroxidation. Following treatments, 2 ml of supernatant from each treatment was aspirated and freeze dried. The lyophilized supernatants were then reconstituted in 500µl of 0.1M phosphate saline buffer (PBS) and used for the TBARS assay. The TBARS assay was conducted as per the method described by Phulukdaree et al., 2010 [Phulukdaree et al., 2010]. Absorbance of the samples was read using a spectrophotometer, λ = 532/600nm (Bio-Tek µQuant, Winooski, VT, USA). MDA concentrations (mM) were calculated by dividing the mean absorbance of the samples by the absorption coefficient (156mM^{-1}).

Intracellular reactive oxygen species

Intracellular ROS (Superoxide: $O_2^{\bullet-}$, hydrogen peroxide, peroxynitrite) was quantified by fluorescence activated cell sorting (FACS) using the fluorescent probe, 2′,7′-dichlorodihydrofluorescein diacetate (H2DCF-DA). Following exposure to NRTIs (24 h, 120 h), 1 x 10^6 HepG2 cells were incubated in phenol red-free media supplemented with 10% foetal calf serum and 10µM H2DCFA for 45 min at 37°C.The probe diffuses into the cell and is retained intracellularly, until it is cleaved by intracellular esterases to the non-fluorescent 2',7'-dichlorodihydrofluorescein (H2DCF). Intracellular ROS oxidize H2DCF to 2',7'-dichlorofluorescein (DCF) which emits fluorescence at 525nm. Stained cells were rinsed thrice with 0.1M PBS (400 x g, 5min, RT) and resuspended in 150µl of 0.1M PBS. Fluorescence of 20 000 events was measured using the FL-1 channel (525nm) of the Accuri™ C6 flow cytometer (BD Biosciences, Franklin Lakes, New Jersey, USA). Live cells were gated using CFlow Plus Software (BD Biosciences, San Jose, CA, USA). Results were reported as percentage DCF positive.

JC-1 Mitoscreen

The percentage mitochondrial depolarisation ($\Delta\psi_m$) was measured using FACS and the JC-1 Mitoscreen (BD Biosciences, San Jose, CA, USA). Briefly, JC-1 stock solution was diluted in 1X Assay Buffer (37°C) to make up a working solution. Approximately 200,000 cells suspended in 100µl of 0.1M PBS from each treatment were transferred to 1.5ml tubes containing 100µl of JC-1 staining solution and incubated (10 min, 37°C). Thereafter, 100µl of FACS sheath fluid was added to each sample. Flow cytometry data from stained cells (50 000 events) was captured with the Accuri™ C6 flow cytometer and software (BD Biosciences, San Jose, CA, USA). Live cells were gated using CFlow Plus Software (BD Biosciences, San Jose, CA, USA).

Reduced and total glutathione

The endogenous antioxidant, reduced glutathione (GSH) was measured by the GSH-Glo™ Glutathione Assay (Promega, Madison, USA) as per the manufacturer's instructions. Both reduced glutathione (GSH) and a total of reduced and oxidized glutathione (GSSG) were quantified. The total amount of glutathione present (GSH+GSSG) was quantified by addition of Tris-(2-carboxyethyl)-phosphine (TCEP) which reduces GSSG to GSH.

Briefly, 6 GSH standards were prepared (0µM - 50µM) to derive a standard curve. 50µl of cell suspension (20,000 cells)/standard solution was aliquot in a white microtitre plate. For the quantification of GSH+GSSG, 10µM of TCEP was added per well. Thereafter, 50µl GSH-Glo™ Reagent (1µl Luciferin-NT, 1µl Glutathione-*S*-transferase, 48µl GSH-Glo™ Reaction buffer) was added per well and incubated for 30 min. Luminescence was measured on a Modulus™ microplate luminometer (Turner BioSystems, Sunnyvale, USA).

ATP

ATP was measured by the luminometric Cell Titer-Glo® assay. Cells were aliquoted in a white microtitre plate (20,000 cells per well) in a 1:2 ratio with ATP Cell Titer-Glo® Reagent (Promega, Madison, USA). The plate was incubated for 30 min at RT. Luminescence was measured on a Modulus™ microplate luminometer (Turner BioSystems, Sunnyvale, USA). Luminescence is proportional to ATP concentration and was expressed as relative light units (RLU).

RNA isolation and quantitative PCR

Complementary DNA (cDNA) was synthesized from RNA for quantitative PCR (qPCR). Total RNA was isolated using an in-house protocol [Chuturgoon et al., 2014]. RNA concentrations were determined (Nanodrop 2000, ThermoScientific, South Africa) and standardised to a concentration of 1, 000ng/µl. RNA was then converted to cDNA using the iScript™ cDNA Synthesis kit (BioRad;

catalog no. 107-8890). A reaction volume of 20µl containing 4µl of 5X iScript reaction mix, 1µl of iScript reverse transcriptase and 2, 000ng of RNA template were made up in nuclease free water. Thermocycler conditions for cDNA synthesis were 25°C for 5 min, 42°C for 30 min, 85°C for 5 min and a final hold at 4°C.

Gene expression of oxidative DNA damage response (*OGG1*) and mitochondrial transcription (*TFAM*) were determined by qPCR. The iQ™ SYBR® Green Supermix (BioRad, 170-880) was used to measure mRNA levels of the genes of interest. Briefly 1.5µl of cDNA template was added to 1µM of sense primer (*TFAM*: 5'-TATCAAGTGCTTATAGGC-3' ;*OGG1*: 5'-GCATCGTACTCTAGCCTCCAC-3'), 1µM antisense primer (*TFAM*: 5'-CACTCCTCAGCACCATATTTCG-3';*OGG1*: 5'-AGGACTTTGCTCCCTCCAC-3'), 5X iScript reaction mix and nuclease free water in a final reaction volume of 25µl. Initial denaturation was conducted at 95°C for 4 min, followed by 37 cycles of denaturation (95°C; 15 sec), annealing [50°C (*TFAM*)/60°C (*OGG1*); 40 sec], and extension (72°C; 30 sec).

All qPCR experiments were conducted on the CFX96 Touch™ Real-Time PCR Detection System (BioRad). Changes to gene expression were calculated according to the methods described by Livak and Schmittgen [Livak, 2001]. Results are converted from mean relative fold change ($2^{-\Delta\Delta Ct}$) to percentage mRNA expression relative to the untreated control (100%). A minimum of three housekeeping genes, β-Actin (sense: 5'-TGACGGGTCACCCACACTGTGCCCAT-3'; antisense: 5'-CTAGAAGCATTTGCGGTGGACGATGGAGGG-3'); GAPDH: (sense: 5'-TCCACCACCCTGTTGCTGTA-3'; antisense: 5'-ACCACAGTCCATGCCATCAC-3'); 18S: (sense: 5'-ACAGGGACAGGATTGACAGA-3'; antisense: 5'-CAAATCGCTCCACCAACCTAA-3') was amplified simultaneously under the same conditions.

Mitochondrial DNA viability

DNA was isolated from cell suspensions as described by Sambrook [Sambrook and Russell, 2001]. Isolated DNA was quantified using the Nanodrop 2000 spectrophotometer and standardized to 5ng/µl. A reaction volume of 25µl consisting of 12.5µl SYBR Green Supermix (BioRad, Hercules, CA, USA), 10*p*mol of forward (5'-TGAGGCCAAATATCATTCTGAGGGC-3') and reverse primer (5'-TGCACCTGCTCTCTGTGATTATGACTATCCCACAGTC-3'), and ~10ng DNA template made up in nuclease free water. Changes in mtDNA application were calculated based on the method described by Livak and Schmittgen, 2001 [Livak, 2001] and reported as percentage mtDNA amplification relative to untreated control (100%). A house keeping gene, β-Globin (sense: 5'-ACATGATTAGCAAAGGGCCTAGCTTGACTCAGA-3'; antisense: 5'-TGCACCTGCTCTGTGATTATGATATCCCACAGTC-3') was amplified simultaneously. Initial denaturation was applied (94°C; 3 min), followed by 28 cycles of denaturation (94°C; 20 sec);

annealing (58°C; 10 min), extension (72°C; 10 min) and a plate read. This was followed by a melt curve and a final hold (25°C; 5 min).

Western blots

Protein expression of PGC-1α, Nrf2, UCP2 and SOD1 were determined using western blots. Briefly, 200µl of Cytobuster (Novagen, San Diego, CA, USA) supplemented with protease and phosphotase inhibitors (Roche, Mannheim, Germany) was added to treated cells in cell culture flasks and kept on ice for 30 min before being scraped and transferred to 1.5ml tubes. The cell lysates were centrifuged (13,000 *g*, 10 min). Crude protein was quantified using the bicinchoninic assay. All protein samples were standardised to 2mg/ml. Laemmeli buffer [dH_2O, 0.5M Tris-HCl (pH 6.8), glycerol, 10% SDS, β-mercaptoethanol, 1% bromophenol blue] was added to the samples (1:1 ratio) and boiled for 5 min. Samples were separated by electrophoresis on a SDS polyacrylamide gel (4% stacking, 10% resolving) for 1 h at 150 V. The separated proteins were transferred to a nitrocellulose membrane using the TransBlot Turbo Transfer System (BioRad, Hercules, CA, USA) at 400mA for 45 min. Membranes were incubated in a blocking solution of 5% bovine serum albumin (BSA) in Tween 20-Tris buffered saline (TTBS) for 1 h; at RT. The membranes were incubated with primary antibody [mouse anti-SOD1 (CS4266), rabbit anti-Nrf2 (ab31163), goat anti-UCP2 (ab77363), rabbit anti-PGC-1α (CS2178), 1: 1 000 dilution in 1% BSA] for 1 h; at RT, and then overnight at 4°C. The membranes were washed five times with TTBS (10 min) before being incubated in HRP-conjugated secondary antibody [goat anti-mouse (31800); goat anti-rabbit (ab6112) 1:10 000 in 1% BSA] for 1 h; at RT. The membranes were then washed 5 times in TTBS (10 min). Clarity Western ECL Substrate (BioRad, Hercules, CA, USA) detection reagent was used to visualise protein bands. Images were captured using gel documentation system Alliance 2.7 (UviTech, Cambridge, UK). Relative band density (RBI) was measured by densitometric analysis using UViTech Alliance Analysis software.

Membranes were stripped with 5% hydrogen peroxide, incubated in blocking solution (3% BSA; 1 h; RT), rinsed twice in TTBS and probed with HRP-conjugated antibody for the house-keeping protein, β-actin (Sigma, St Louis, MO, USA). The relative band intensity was normalised against β-Actin and then reported as fold change relative to the control.

Statistical analysis

Statistical analyses were performed using the GraphPad Prism V5 software package (GraphPad Software Inc., San Diego, California, USA). All data sets were assessed for Gaussian distributions using the D'Agostino & Pearson omnibus normality test. Comparisons between the control and drug treatments were done by performing Kruskal-Wallis tests (nonparametric distributions).

Results

Oxidative damage and intracellular antioxidant levels

HepG2 cells exposed to AZT and TFV for 24 h had higher levels of intracellular ROS (Figure 3.1A; $p = 0.02$) and MDA levels ($p = 0.02$; Figure 3.1B) relative to the untreated control. In the same time period, no significant changes were observed in the intracellular ROS and MDA levels of cells exposed to d4T ($p>0.05$). Following 120 h exposure, all three drugs increased both intracellular ROS (Figure 1A) and extracellular MDA levels (Figure 3.1B) compared to the control. Intracellular ROS levels were significantly higher in the TFV treated cells at 120 h ($p = 0.02$), and d4T caused the greatest increase in MDA levels ($p = 0.02$).

At 24 h, base excision repair response, *OGG1*, was down-regulated in all treatments, most so in the AZT and d4T treatment ($p < 0.0001$; Table 3.1). However, after 120 h, all three NRTIs increased *OGG1* mRNA levels relative to the control (Table 3.1). Most notably there was a 10-fold increase in the d4T and TFV treatments ($p = 0.0003$).

After 24 h exposure, AZT increased both GSH ($p = 0.04$, Figure 3.1C) and GSH+GSSG ($p = 0.02$, Figure 3.1D) levels relative to the control. In the same time period, d4T reduced both GSH (Figure 3.1C) and GSH+GSSG (Figure 3.1D). HepG2 cells exposed to TFV for 24 h showed lowered GSH levels (Figure 3.1C) but GSH+GSSG levels remained similar to the control (Figure 3.1D). After 120 h, GSH levels were higher in the NRTI treated HepG2 cells than the untreated control. This, however, was not statistically significant ($p = 0.06$; Figure 1B). Total glutathione (GSH+GSSG) was only increased by TFV at 120 h ($p = 0.02$; Figure 3.1D), whilst AZT and d4T decreased GSH+GSSG relative to the control.

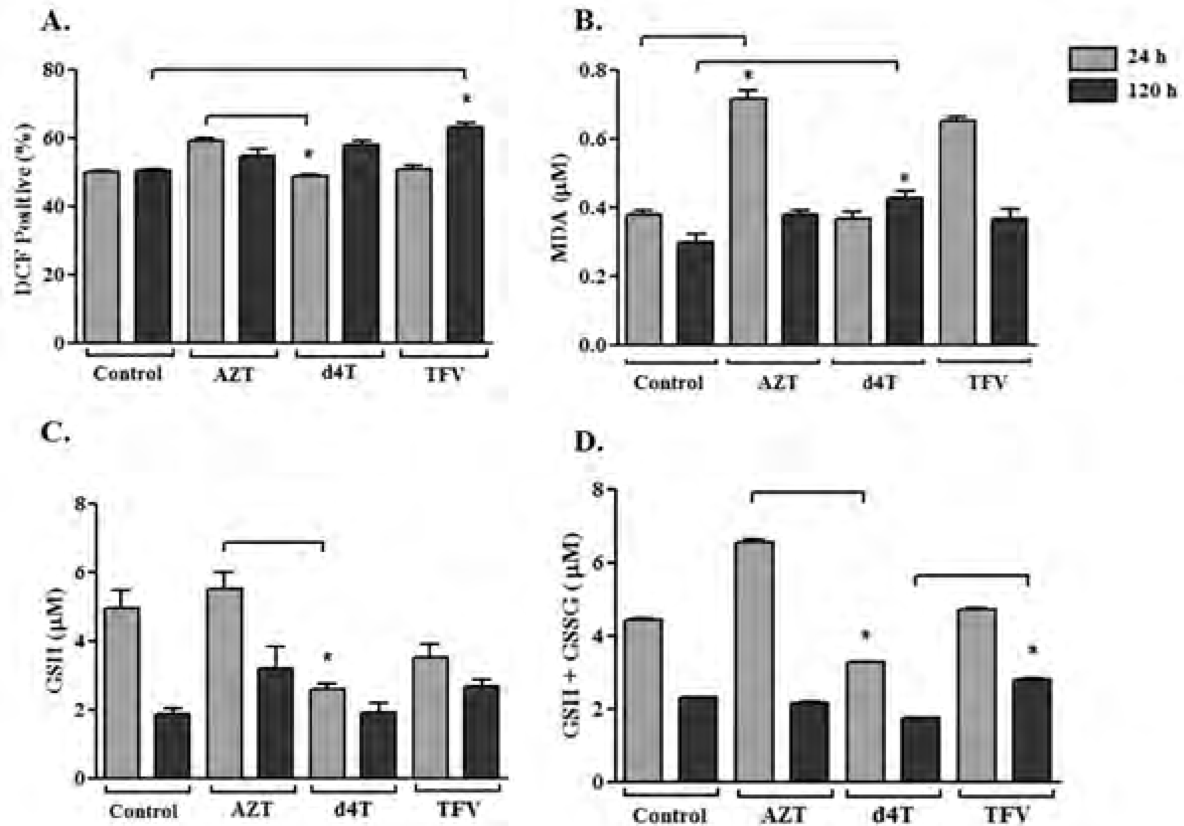

Figure 3.1: Intracellular reactive oxygen species measured as DCF fluorescence (A), extracellular malondialdehyde levels (B), GSH (C) and total glutathione (GSH+GSSG; D) in HepG2 cells treated with NRTIs at 24 h and 120 h, *$p < 0.05$ relative to control

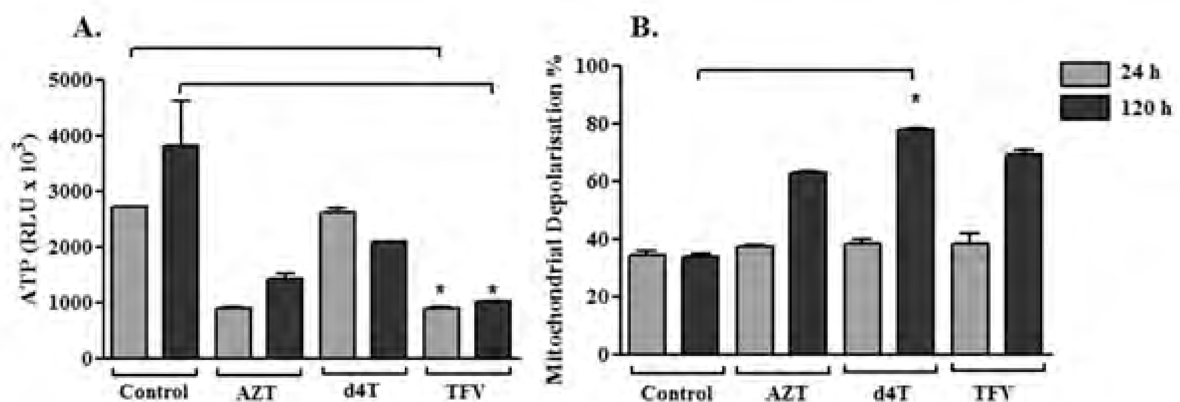

Figure 3.2: ATP levels (A) and mitochondrial (mt) depolarisation (B) in HepG2 cells treated with Zidovudine (AZT), Stavudine (d4t) and Tenofovir (TFV) at 24 h and 120 h, *$p < 0.05$ compared to untreated control. RLU: relative light units

Mitochondrial integrity and function

Mitochondrial membrane depolarisation and ATP were measured as mt function parameters. At both time periods, AZT and TFV markedly reduced ATP levels (24 h: p = 0.0.1; 120 h: $p = 0.02$, Figure 3.2A) with a concomitant increase in $\Delta\psi_m$ (24h: $p = 0.12$; Figure 3.2B; 120 h: $p = 0.02$; Figure 3.2B). ATP levels remained unchanged at 24 h in the d4T treatment, but a marked decline was observed at 120 h ($p < 0.05$). Percentage $\Delta\psi_m$ was elevated in HepG2 cells exposed to d4T for 24 h, but not significantly ($p = 0.02$; Figure 3.2B).

The mt response to $O_2^{\bullet -}$ production was evaluated by quantifying UCP2 expression. At 24 h, UCP2 protein expression increased in the AZT [1.75 RBI (Inter-quartile range: IQR: 1.70, 1.81)] and d4T treatment [1.80 RBI (IQR: 1.60, 2.18)] relative to the control [1.49 RBI (IQR: 1.26, 1.74); Figure 3.3]. The 120 h treatment showed an increase in UCP2 expression in the NRTI treated cells [Control: 1.37 RBI (IQR: 1.20, 1.58); AZT: 1.53 RBI (IQR: 1.42, 1.68); d4T: 1.74 RBI (IQR: 1.63, 1.93)], with a significant increase in the TFV treatment [2.02 RBI (IQR: 1.87, 2.15); $p = 0.04$; Figure 3.3].

Protein expression of PGC-1α, a regulator of mt biogenesis, was assessed as a response to mt damage, while *TFAM* mRNA levels evaluated mtDNA transcription. The qPCR results showed that *TFAM* mRNA levels at 24 h was not differentially regulated in the TFV and AZT treatments (Table 3.1). Stavudine, however, significantly reduced *TFAM* mRNA levels at 24 h ($p = 0.003$; Table 3.1). Following 120 h exposure, *TFAM* mRNA levels increased in all NRTI treated HepG2 cells exceeding 2 fold ($p < 0.0001$; Table 3.1). Expression of PGC-1α protein was also elevated by all three NRTIs at both 24 h [AZT: 3.24 RBI (IQR: 3.16, 3.33); d4T: 4.44 RBI (IQR: 4.21, 4.76)] and 120 h [AZT: 2.7 1RBI (IQR: 2.51, 2.91); d4T: 2.94 RBI (IQR: 2.30, 3.40)] compared to their respective controls [Control 24 h: 2.57 RBI (IQR: 2.19, 2.96); Control 120 h: 2.01 RBI(IQR: 1.89, 2.10)]. Tenofovir induced significantly higher expression of PGC-1α in both time periods [24 h: 7.38 RBI (IQR: 6.69, 8.11); $p = 0.02$; 120 h: 4.32 RBI (IQR: 4.0, 4.63); $p = 0.02$; Figure 3.3].

The amount of viable mtDNA in each treatment was determined by qPCR. This assay works on the principle that viable mtDNA levels will be proportional to mtDNA amplification. The AZT and TFV treatments caused an increase in mtDNA amplification relative to the control following 24 h exposure (Table 3.1). The d4T treatment, however, decreased the amount of viable mtDNA in this time period ($p = 0.04$; Table 3.1). At 120 h, AZT also caused a decline in mtDNA integrity and a significant decrease was observed in the d4T treatment ($p = 0.01$). Tenofovir still exhibited increased mtDNA amplification at 120 h (Table 3.1).

Table 3.1: Percentage regulation analysis of mRNA expression (*OGG1*; *TFAM*) and mtDNA amplification relative to untreated control at 24 h and 120 h treatment with NRTIs

		Viable mtDNA	*TFAM*	*OGG1*
24 h	**AZT**	184 (168, 211)	105 (51.1, 169)	44.5 (36.5, 60.3)*
	d4T	69.56 (53.6, 84.9)	6.37 (4.51, 9.19) **	2.98 (1.68, 3.69)***
	TFV	151 (95.2, 251)	98.1 (82.8, 106)	56.7 (53.9, 58.23)
120 h	**AZT**	76.1 (65.3, 83.3)	830 (748, 942)***	104 (982, 117)
	d4T	36.1 (15.0, 76.4)*	278 (264, 304)	98.1 (82.8, 106)**
	TFV	189.4 (144, 214.6)	633 (557, 740)*	1047 (980, 1170)***

Data represented as median (IQR) *$p < 0.05$; *$p < 0.005$, ***$p < 0.0001$ relative to control (100%), AZT: zidovudine, d4t: stavudine, TFV: tenofovir, mt: mitochondrial, TFAM: mitochondrial transcription factor A, OGG1: 8-oxoguanine DNA glycosylase

Antioxidant response

The expression of key AO proteins was measured to determine the AO response induced by NRTIs. At 24 h, only TFV significantly increased Nrf2 protein expression in HepG2 cells [8.40 RBI (IQR: 7.05, 9.28); $p = 0.01$; Figure 3.3] compared to the control [5.26 RBI (IQR: 5.02, 5.50); Figure 3.3]. At 120 h, however, the expression of Nrf2 was elevated by d4T [5.04 RBI (IQR: 4.63, 5.82)] and AZT treatments [4.68 RBI (IQR: 3.86, 5.36)] relative to the control [2.83 RBI (IQR: 2.49, 3.30)], and significantly up-regulated by TFV [7.22 RBI (IQR: 6.83, 7.88); $p = 0.02$; Figure 3.3].

SOD1regulates both mitochondrial and cytosolic detoxification of $O_2^{\bullet-}$. Only TFV significantly up-regulated protein expression of SOD1 at 24 h [0.34 RBI (IQR: 0.33, 0.35); Figure 3.3] and 120 h [6.35 RBI (IQR: 6.15, 6.72); $p = 0.02$; Figure 3.3] compared to the control (Figure 3.3). Stavudine [24h: 0.02RBI (IQR: 0.02, 0.02); 120 h: 4.18RBI (IQR: 3.54, 4.50)] and AZT [24 h; 0.02RBI (IQR: 0.01, 0.02); 120h: 4.33RBI (IQR: 3.36, 5.51)] also induced higher SOD1 expression ($p > 0.05$).

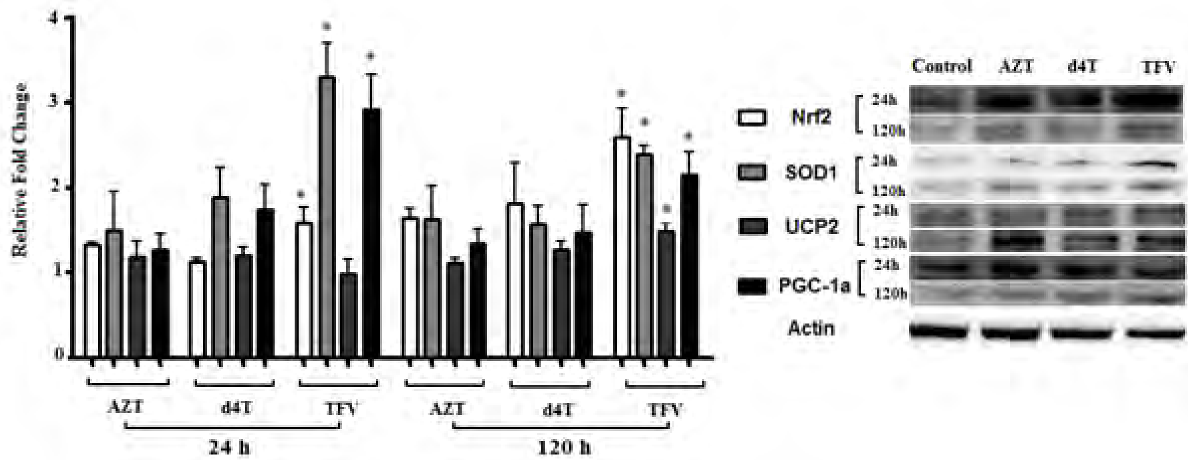

Figure 3.3: Densitometry analysis and western blot images for antioxidant and mitochondrial oxidative stress response protein expression at 24 h and 120 h treatment with NRTIs. *$p < 0.05$ relative to control

Discussion

Liver toxicity is commonly observed in patients on long term antiretroviral therapy. No clear mechanism has been distinguished as individual ARVs have varying degrees of toxicity [Núnez, 2006]. Stavudine and AZT are considered among the most hepatotoxic NRTIs [Ter Hofstede et al., 2000; Wit et al., 2002] with TFV exhibiting very low hepatic toxicity [Birkus et al., 2002]. Hepatocytes possess cytoprotective mechanisms against xenobiotic insult. In this study, we assessed the mitochondrial and AO stress responses to NRTIs in liver cells after 24 h and 120 h exposure.

The involvement of Nrf2 in the defence against NRTI-associated toxicity integrates two major mechanisms of NRTI toxicity – oxidative stress and mt toxicity. Firstly Nrf2 regulates the transcription of AO genes during oxidative stress response [Motohashi and Yamamoto, 2004]. Secondly, the ARE to which Nrf2 binds in the nucleus shares motifs with transcription factor, NRF1 [Piantadosi et al., 2008]. This provides an overlap in the function of Nrf2 and PGC-1α, as both promote transcription of mtDNA via activation of NRF1. We observed that NRTIs increased Nrf2 and PGC-1α protein expression, most so at 120 h (Figure 3.3). Increased PGC-1α expression by NRTIs was also accompanied by lower ATP levels (Figure 3.2A). Reduced ATP, an indication of reduced mt function, favours the activation of the AMP-activated protein kinase pathway, which in turn increases mt biogenesis via PGC-1α and TFAM activity [Zong et al., 2002]. The upregulation of PGC-1α induced by NRTIs would not only promote mt biogenesis, but also increase the transcription of mt defence genes including manganese SOD and UCP2 [Valle et al., 2005] in response to mt oxidative stress.

Of the NRTIs evaluated in our study, TFV elicited the highest AO response, with a significant increase in the translational regulation of Nrf2 and its downstream target, SOD1 (Figure 3.3). Tenofovir is considered one of the least toxic NRTIs and is commonly used in pre-exposure prophylaxis for HIV transmission prevention [Celum and Baeten, 2012]. At 120 h TFV increased intracellular ROS (Figure 3.1A) indicating early signs of mt oxidative stress. However, the strong antioxidant response in the TFV treated cells dampens oxidative damage so that lipid peroxidation is lower compared to AZT and d4T treated cells (Figure 3.1B).

A previous study evaluating the cytotoxicity of TFV on HepG2 cells found that of five NRTIs tested, including AZT and d4T, TFV exhibited weak hepatic toxicity [Cihlar et al., 2002]. Furthermore, TFV is a weak inhibitor of DNA polymerase γ [Lewis et al., 2003], the enzyme responsible for mtDNA replication, and does not affect mtDNA content in HepG2 cells [de Baar et al., 2007]. Our study was consistent with this finding, as mtDNA levels were not depleted following 120 h exposure to TFV (Table 3.1). However, we still found markers for mt dysfunction in TFV treated HepG2 cells (Figure 3.2A; 3.2B). Studies assessing mt toxicity in TFV-induced nephropathy reported mt swelling and structural changes to mitochondria [Abraham et al., 2013; Kohler et al., 2009]. Our present study found that TFV caused a shift in mt membrane potential (Figure 3.2B) which would compromise the integrity of the mt membrane. This can change the permeability of the mt membrane, promoting mt swelling. This mechanism of mt toxicity can occur independently of mtDNA depletion.

Thymidine analogues, AZT and d4T, are inhibitors of DNA polymerase γ [Bienstock and Copeland, 2004] and can therefore disrupt mtDNA replication. Various studies have investigated the effect of these two NRTIs on mt function and mtDNA in different cell lines with conflicting results. This suggests that AZT and d4T toxicity is tissue-specific and exposure time dependent. A study conducted by Banerjee et al. in 2013 reported AZT significantly increased oxidative stress markers in rat liver homogenates following acute exposure [Banerjee et al., 2013]. Another study comparing chronic exposure of endothelial cells to AZT and d4T found AZT increased oxidative stress and mt dysfunction, and not d4T [Kline et al., 2009]. However, work by Birkus et al. (2002) on HepG2 cells showed d4T was more toxic to mitochondria than AZT [Birkus et al., 2002]. Research by Velsor et. al (2004) on HepG2 cells corroborated that d4T exhibits mt toxicity and also induced mt oxidative stress [Velsor et al., 2004]. Our present work found AZT induced oxidative stress during early exposure (24 h) but markers for oxidative stress began to decline following 120 h exposure. Stavudine, however, only induced oxidative damage following 120 h exposure. The significantly lowered GSH levels in the d4T treated HepG2 cells (Figure 3.1C) is likely to contribute to the elevated oxidative damage observed in this treatment. Both drugs exhibited mitochondrial toxicity (Figure 3.2A, 3.2B), but this was more severe in the d4T treated cells. As d4T is the more potent inhibitor of DNA polymerase γ, and continually decreased mtDNA levels over time (Table 3.1), this may be a contributing factor to d4T being more toxic than AZT.

Conclusion

Our study found that NRTIs induced both the Nrf2 and PGC-1α stress responses, and this was highest in the TFV treatment. All drugs exhibited mt toxicity, especially at 120 h of exposure. Tenofovir induced mt dysfunction without reducing mtDNA levels. The d4T treatment showed the highest markers for oxidative stress at 120 h and lowest levels of mtDNA viability in HepG2 cells. We can conclude that Nrf2 and PGC-1α are very likely to play a role in the mt and oxidative stress response to NRTIs. Comparatively, d4T is the most likely NRTI to exhibit hepatotoxicity following prolonged exposure.

Declaration of Interest

None declared

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CHAPTER FOUR

Lon protease and eiF2α kinase are involved in acute, but not prolonged, antiretroviral induced stress response in HepG2 cells

Savania Nagiah, Alisa Phulukdaree [1], Anil A Chuturgoon [a]

[a] Discipline of Medical Biochemistry, School of Laboratory Medicine and Medical Sciences, College of Health Science, University of KwaZulu Natal, Durban, South Africa

[1] Present address: Department of Physiology, Faculty of Health Sciences, Prinshof Campus, University of Pretoria, Pretoria, South Africa

Address and contact information:

Prof Anil Chuturgoon (corresponding author): 3rd Floor George Campbell Building, Discipline of Medical Biochemistry, Howard College, University of KwaZulu Natal, King George Avenue, Durban, 4041, South Africa; **email: CHUTUR@ukzn.ac.za**

Savania Nagiah: 3rd Floor George Campbell Building, Discipline of Medical Biochemistry, Howard College, University of KwaZulu Natal, King George Avenue, Durban, 4041, South Africa; **email: 208509769@ukzn.stu.az.za**

Dr Alisa Phulukdaree: Department of Physiology, School of Medicine, Prinshof Campus, Dr Savage Road, 0083 Prinshof, Pretoria, Gauteng, South Africa; **email: phulukdaree@up.ac.za**

Chemico-Biological Interactions

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[1] **Abbreviations:** AAA+- ATPases associated with diverse cellular activities; AZT - Zidovudine; CHOP - CCAAT-enhancer-binding protein homologous protein; d4T - Stavudine; eIF2α - eukaryotic translation initiation factor 2α; ER – endoplasmic reticulum; HSP - heat shock protein; JNK – c-jun N-terminal kinase; mt – mitochondrial; NRTI – nucleoside reverse transcriptase inhibitor; PERK – protein kinase RNA-like ER kinase; RBI – relative band intensity; SIRT3 – Sirtuin 3; TFV: Tenofovir

Abstract

Lon protease, an ATP dependent mitochondrial (mt) protease, is important in mt protein maintenance. Disruption of protein homeostasis and mt dysfunction is associated with lipodystrophy, metabolic syndrome and accelerated aging, and are commonly observed in patients on long term antiretroviral therapy. Sirtuin 3 (SIRT3) is a post-translational regulator of Lon and regulates antioxidant response. We previously showed the nucleoside analogues (NRTIs), Zidovudine (AZT; 7.1µM), Stavudine (d4T; 4µM), and Tenofovir (TFV; 1.2µM) induced oxidative stress and mt dysfunction in human hepatoma (HepG2) cells at 24 hours (h) and 120 h. We conducted a mt proteomic assessment of homeostasis in the same model, using the same NRTIs. Protein expression of Lon, SIRT3, heat shock protein (HSP) 60, phospho-eukaryotic translation initiation factor 2α (p-eIF2α; Ser51) and phospho-c-jun N-terminal kinase (p-JNK; Thr183/Tyr185) were quantified by western blots. The data showed all stress responses were significantly increased in HepG2 cells by all antiretroviral drugs at 24 h ($p<0.0001$); however, at 120 h, a significant depletion in the ATP-dependent proteins Lon ($p=0.00013$) and HSP60 ($p<0.0001$) was observed. Proteins initiated by endoplasmic reticulum stress: p-eIF2α (p=0.001) and p-JNK ($p=0.0029$), were significantly reduced following prolonged treatment. SIRT3 was maintained at elevated levels in the treated cells following prolonged exposure ($p<0.001$). We conclude that the ATP dependent proteins are more relevant to acute toxicity, while SIRT3 confers protection over prolonged periods of toxicity.

Key words: NRTI; Lon; SIRT3; eIF2α; mitochondrial stress

Introduction

Mitochondrial (mt) integrity is imperative to the optimal functioning of a biological system. Functionality of mitochondria is maintained by tightly regulated stress and repair mechanisms. A progressive decline in the efficacy of these repair and stress responses is a characteristic of biological aging (Hamon et al., 2015). The result of impaired stress responses is reduced mt respiratory capacity and increased production of free radicals, leading to oxidative stress. The highly oxidative environment created makes protein dense mitochondria susceptible to oxidative modification(s). Oxidatively modified proteins, if not cleared by proteolysis, begin to form occlusions and may result in aberrant structural changes to mitochondria (Ngo et al., 2013). The disruption to protein homeostasis in the mitochondria elicits cellular stress responses to recruit repair mechanisms and mt chaperones.

Cellular proteases and chaperones are essential for the removal of misfolded or aberrant proteins. Proteolytic removal of oxidized proteins from the mt matrix is largely achieved by the evolutionarily conserved Lon protease (Lon). This mt protein, encoded for by the gene *LONP1*, belongs to the ATPases Associated with diverse cellular Activities (AAA+) protease family. Lon has the ability to recognise misfolded or damaged proteins by the exposed hydrophobic core of target proteins. The damaged protein is then proteolytically processed by Lon in an ATP-dependent manner (Gur and Sauer, 2008). Substrates of Lon are not exclusive to aberrant or misfolded proteins. The role of Lon in mtDNA turnover has been identified due to its selective degradation of mitochondrial transcription factor A (Lu et al., 2013; Matsushima et al., 2010). The central role of Lon in mt protein maintenance indicates the potential of this protein in many diseases related to mt dysfunction (Ngo et al., 2013).

Hypoxia, oxidative stress, endoplasmic reticulum (ER) stress and ATP availability are inducers of Lon expression. Lon is involved in acute oxidative stress response, however, its activity cannot be maintained during chronic stress (Ngo et al., 2013). There have been no correlations found between *LONP1* mRNA levels and Lon protein expression, suggesting posttranscriptional regulation of this protein (Gibellini et al., 2014). This is common in multiple proteins involved in stress response as it provides a means to increase protein turnover without the time consuming synthesis, processing and exportation of *de novo* mRNA synthesis. The posttranslational regulation of Lon by Sirtuin 3 (SIRT3) was recently described by Gibellini et al. (2014). Silencing of SIRT3 in breast cancer cells led to an accumulation of Lon protein but not *LONP1* mRNA.

The sirtuin family of NAD^+ dependent deacetylases has been gaining attention for their role in longevity and metabolism. The mt localized SIRT3 is responsible for fat metabolism, antioxidant defence (Hirschey et al., 2010), mt integrity and function, and the mt unfolded protein response (UPR^{mt}) (Papa and Germain, 2014). The function of SIRT3 in maintaining protein homeostasis is independent of the activity of the transcription factors CHOP and oestrogen receptor α. This new

mechanism of SIRT3 mediated UPR^{mt} is believed to be mediated by SIRT3 antioxidant and mitophagy regulation (Papa and Germain, 2014). A depletion of SIRT3 is associated with accelerated aging-associated disorders (McDonnell et al., 2015). The overlapping role of SIRT3 and Lon in the maintenance of protein homeostasis is a scarcely described mechanism in acute and chronic stress response.

Studies have shown Lon interacts with other mt chaperones including heat shock protein (HSP) 60, and mtHSP70 (Hori et al., 2002; Kao et al., 2015). Cellular chaperones such as HSPs confer resistance to stress conditions, promoting cell survival. The complex formed by Lon, HSP60 and mtHSP70 has been identified as a component in the transmission of ER stress to the mitochondria. Prolonged oxidative stress causes an increase in misfolded proteins which aggregate in the ER. The objective of the ER stress response is to inhibit or slow down protein synthesis, reducing the load of client proteins in the ER. Translational repression is achieved by phosphorylation of eukaryotic translation initiation factor 2α (eIF2α) kinase. This protein is activated by protein kinase RNA-like ER kinase (PERK) or c-jun N-terminal kinase (JNK). Phosphorylation of eIF2α kinase is elevated during oxidative stress and has been suggested as an ER response to mt stress (Baker et al., 2012).

The long term use of nucleoside reverse transcriptase inhibitors (NRTIs) in antiretroviral therapy is associated with metabolic syndrome, lipodystrophy (Carr and Cooper, 2000) and accelerated aging (Smith et al., 2012). Mitochondrial dysfunction has been implicated as a molecular mechanism for these clinical outcomes (Brinkman et al., 1999). Mitochondrial protein maintenance plays a critical role in retarding the development of NRTI-associated adverse health outcomes. Presently, only one study has quantified Lon expression in relation to antiretroviral use and lipodystrophy (Pinti et al., 2010), however the response of SIRT3 and Lon in NRTI-induced hepatotoxicity has not been described. We previously showed that the NRTIs Zidovudine (AZT) and Tenofovir (TFV) induced acute oxidative stress in human hepatoma (HepG2) cells, while Stavudine (d4T) and TFV elevated oxidative stress markers following prolonged exposure (Nagiah et al., 2015). This was accompanied by a heightened antioxidant response. We replicated this model to assess the role of Lon and SIRT3 in the mt protein homeostatic response in HepG2 cells following acute [24 hour (h)] and prolonged (120 h) exposure to NRTIs: AZT, d4T and TFV.

Methods

Cell culture and treatment

Human hepatoma (HepG2) cells were cultured in $25cm^3$ cell culture flasks in Eagles minimum essential medium (Lonza; BioWhittaker, Johannesburg, South Africa) supplemented with 10% foetal calf serum, 1% L-glutamine and 1% penstrepfungizone. Cells were allowed to reach 80% confluence prior to treatment. Stocks of antiretroviral drugs were made in DMSO (10mM). Cells were treated

with maximum plasma level concentrations (Venhoff et al., 2007; Walker et al., 2002) of AZT (7.1μM); d4T (4μM); and TFV (1.2μM). Antiretroviral drugs used were obtained from the NIH AIDS reagents program. Cells were exposed to NRTIs for 24 hours (h) and 120 h prior to harvesting for protein extraction.

Protein preparation

The protein expression of key proteins involved in mt protein homeostasis was assessed by the western blot technique. Treated cells were rinsed twice with 0.1M phosphate saline buffer (PBS) and incubated in 200μl Cytobuster reagent (Novagen, San Diego, CA) supplemented with protease and phosphatase inhibitors (Roche, Mannheim, Germany) for 10 minutes (min) on ice. The cell lysates were transferred to 1.5ml micro-centrifuge tubes and centrifuged (13,000 x *g*; 10 min). Crude protein extracts were aspirated and quantified using the bicinchoninic acid assay and standardized to 1.2mg/ml. Proteins samples were then boiled in Laemelli buffer [dH_2O, 0.5M Tris-HCl (pH 6.8), glycerol, 10% SDS, β-mercaptoethanol, 1% bromophenol blue] for 5 min.

SDS-PAGE and Western blot

The prepared protein samples were separated on SDS polyacrylamide gels (4% stacking, 7.5% resolving) by electrophoresis (150 V; 1 h). Separated proteins were transferred to nitrocellulose membranes using the TransBlot Turbo Transfer system (Bio-Rad, Hercules, CA, USA) at 400mA for 45 min. Following transfer, nitrocellulose membranes were incubated in blocking solution (5% non-fat dry milk; 1 h; RT) on a shaker. Membranes were then incubated in primary antibody (1:1000 dilution in 5% non-fat dry milk) for 1 h, at RT on a shaker, followed by overnight incubation at 4ºC. Antibodies were obtained from Cell Signalling Technology (Beverly, MA, USA) and Sigma Aldrich (St Louis, MO, USA): anti-LONP1 (HPA002034), anti-HSP60 (D307), anti-SIRT3 (C73E3), anti-phospho-eiF2α (Ser51; #3597), anti-phospho-JNK (Thr183/Tyr185; #9251). Following probing with primary antibody, membranes were rinsed (5 x 10 min) with Tween 20-Tris buffered saline (TTBS). The membranes were then incubated in horse radish peroxidase-conjugated secondary antibody (1: 10,000 dilution in 5% non-fat dry milk) for 1 h, at RT on a shaker. The membranes were rinsed again (5 x 10 min) in TTBS prior to chemiluminescent detection.
Protein bands were visualized using Clarity Western ECL detection reagent (Bio-Rad, Hercules, CA) and images were captured on the Alliance 2.7 gel documentation system (UviTech, Cambridge, UK). Densitometric analysis was done using the UviBand Analysis software (UviTech, Cambridge, UK). Protein expression was reported as relative band intensity (RBI) normalized against house-keeping protein β-Actin. Membranes were incubated in 5% hydrogen peroxide (37ºC) following detection of proteins to quench the bound secondary antibody. The membranes were then incubated with HRP-conjugated anti-β-Actin (Sigma-Aldrich, St Louis, MO, USA) and rinsed prior to detection. The results reported are the RBI of the protein of interested divided by the RBI of β-Actin.

Statistical analysis

All experiments were conducted independently in triplicate. Statistical analyses were performed using the GraphPad Prism V5 software package (GraphPad Software, Inc., San Diego, CA, USA). All data sets were assessed for Gaussian distributions using the D'Agostino & Pearson omnibus normality test. Comparisons between the control and drug treatments were done by performing Kruskal–Wallis tests (nonparametric distributions).

Results

Mitochondrial chaperone recruitment during NRTI toxicity

The mt chaperones and proteases are required for mt maintenance. These proteins are recruited as part of the UPR^{mt} and during ER stress. The major mt protein chaperone HSP60 was significantly increased by all three NRTIs evaluated at 24 h (AZT: 4.95±0.35RBI; d4T: 2.85±0.10RBI; TFV: 4.99±0.18RBI) compared to the control (1.44±0.08RBI, Figure 4.1; $p < 0.0001$). This was also accompanied by a concurrent significant increase in Lon protein levels (AZT: 0.82±0.05RBI; d4T: 0.93±0.02RBI; TFV: 0.85±0.01RBI) relative to the untreated cells (0.77±0.002RBI; Figure 4.1; $p < 0.001$). Prolonged exposure to NRTIs caused a change in the recruitment of these proteins. The ATP dependent Lon and HSP60 proteins were significantly depleted at 120 h (Figure 4.2, $p < 0.0001$).

SIRT3 induction in NRTI treated HepG2 cells

Sirtuin 3 is a NAD^+ dependent mt deacetylase. The deacetylation of mt proteins is important in post-translation regulation. This function has made SIRT3 central to the antioxidant response and UPR^{mt}. Proteomic assessment of SIRT3 in HepG2 cells treated with NRTIs showed that the thymidine analogues - AZT and d4T significantly elevated SIRT3 at both 24 h (AZT: 0.64±0.01RBI; d4T: 0.57±0.03RBI; Figure 4.1) and 120 h (AZT: 1.08±0.04RBI; d4T: 0.92±0.01RBI; Figure 4.2) relative to the control (24 h: 0.51±0.02RBI; 120 h: 0.85±0.02RBI; $p < 0.0001$). Tenofovir significantly increased SIRT3 protein levels during the acute time period (0.74±0.004RBI), but this was not sustained over prolonged exposure (Figure 4.2; $p > 0.05$).

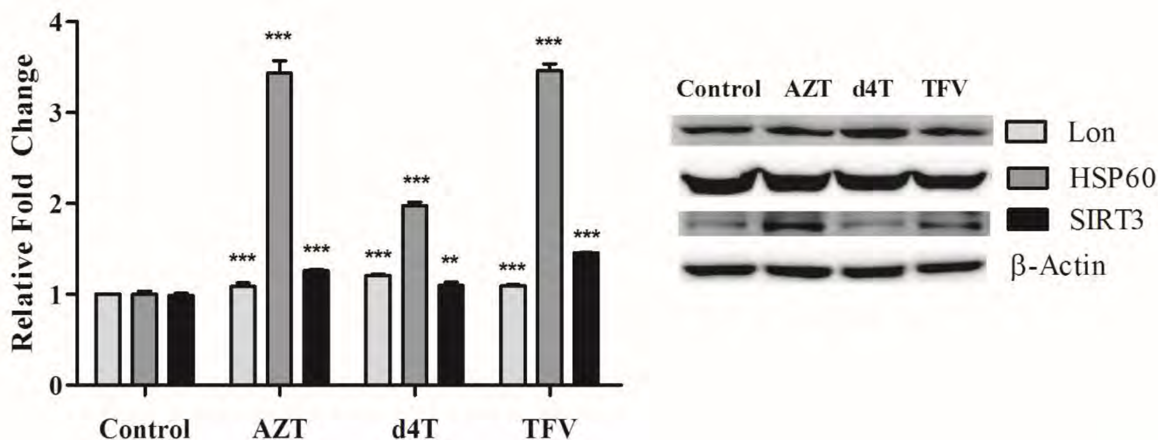

Figure 4.1: Relative band intensity reported as fold change relative to control and western blot images for protein expression of Lon, HSP60 and SIRT3 following acute (24 h) treatment with antiretroviral drugs. ***$p < 0.005$ ****$p < 0.0001$ relative to control

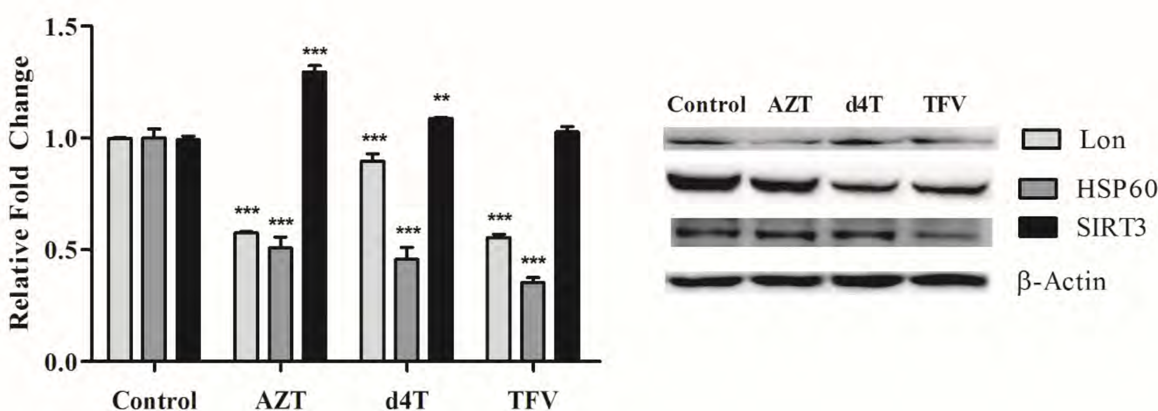

Figure 4.2: Protein expression reported as relative fold change and western blot images for Lon, HSP60 and SIRT3 following 120 h exposure to antiretroviral drugs. ***$p < 0.005$; ****$p < 0.0001$ relative to control

Endoplasmic reticulum stress associated proteins

Phosphorylated JNK (Thr183/Tyr185) and phosphorylated eiF2α were assessed as responses to ER stress. Phosphorylated JNK is a kinase known to activate eiF2α in the event of ER stress. In both time periods there are similar expression patterns in the two proteins in each NRTI treatment. Both proteins were significantly up-regulated following acute exposure to AZT (p-eiF2α: 1.01±0.03RBI; p-JNK: 1.31±0.30RBI) and TFV (p-eiF2α: 0.94±0.09RBI, p-JNK: 0.79±0.07RBI) compared to the untreated control (p-eiF2α: 053±0.02; $p < 0.0001$; p-JNK: 0.46±0.002RBI; $p = 0.0003$; Figure 4.3). At 120 h a significant depletion in p-eiF2α (0.78±0.02RBI; $p = 0.001$) and p-JNK (0.51±0.05RBI; $p = 0.003$) was

observed in the TFV treated HepG2 cells (Figure 4.4). There was also a significant decrease of p-JNK induced by d4T (0.54±0.02RBI) in this time period relative to the control (1.27±0.40RBI).

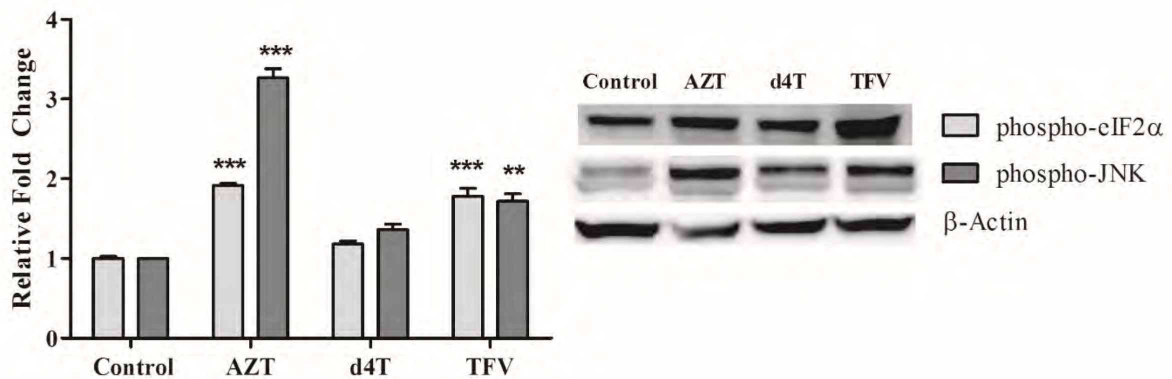

Figure 4.3: Densitometric analysis and western blot images of endoplasmic reticulum stress associated proteins in HepG2 cells following 24 h exposure to antiretroviral drugs. Results are normalized against β-Actin and reported as relative fold change. **p < 0.005; ***p < 0.0001 relative to control

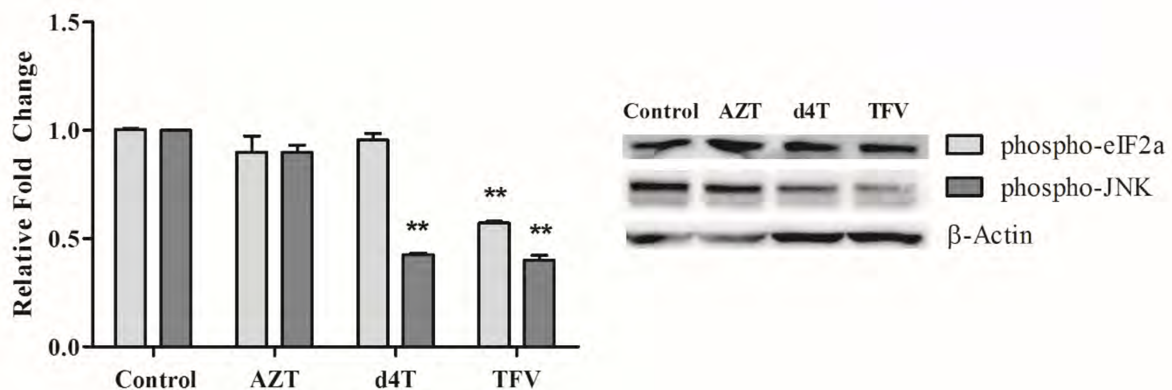

Figure 4.4: Protein expression of endoplasmic reticulum stress associated proteins in HepG2 cells reported as fold change following 120 h exposure to antiretroviral drugs. **p < 0.005 relative to control

Discussion

Proteins have unique three-dimensional structures that are essential for their specific functions. Aerobic metabolism by cells constantly exposes the cells to various oxidizing species that can change the native structure of proteins. The accumulation of cross-linked protein aggregates and the inability of cells to remove protein occlusions eventually become toxic to the cell (Ngo et al., 2013). Mitochondria are protein dense and high traffic organelles exposed to high levels of oxidants. The

reduced capacity to maintain mt function by proteolytic clearance of protein aggregates may be a contributor to metabolic and age-related disorders.

Lipodystrophy, metabolic syndrome and age-associated disorders are common health outcomes with NRTI use (Carr and Cooper, 2000; Smith et al., 2012). Mitochondrial toxicity is an underlying mechanism in all of these adverse drug reactions. Lon and SIRT3 are key components in the UPR^{mt}. Compromised UPR^{mt} with long term use of NRTIs may be a potential mechanism of toxicity and thus we investigated the proteins involved in UPR^{mt} in relation to acute and chronic oxidative stress induced by NRTIs in HepG2 cells.

The relationship between antiretroviral associated lipodystrophy and Lon was first described by Pinti et al. (Pinti et al., 2010). Adipose tissue from patients with lipodystrophy on antiretroviral therapy had significantly elevated gene expression of *LONP1*. It was established in a liposarcoma cell line that Lon expression was highest in d4T treated cells in response to oxidative stress. To date, this was the only study that evaluated Lon and antiretroviral toxicity. Using a hepatic *in vitro* model, we show Lon induction as an acute response to oxidative stress induced by NRTIs (Figure 4.1). This response was not observed in chronic exposure to NRTIs (Figure 4.2). Our data is consistent with that of Pinti et al. as d4T elicited the highest Lon expression at 24 h. Ngo. et al reported Lon as an acute stress response protein, while chronic stress results in dysregulation of Lon (Ngo et al., 2013). This dysregulation is associated with accelerated aging and associated disorders. We hypothesized that Lon, being an ATP dependent protease, could not be sustained over chronic oxidative stress periods resulting in progressive mt failure. In our previous study we showed severe depletion of ATP by the three NRTIs at 120 h. This depleted ATP level strongly correlates with significantly decreased Lon protein levels at 120 h (Figure 4.3). This suggests that continuous proteolytic activity may be detrimental to the cell over a long period of time.

Another indication of ATP availability influencing stress responses is the decrease in HSP60 protein expression at 120 h (Figure 4.3). HSP60 is located in the mt matrix and is one of the most important mt chaperones. It is responsible for the transport of linear proteins from the cytoplasm to the mt matrix and refolding of imported mt proteins (Bukau and Horwich, 1998). The stability of HSP60 has been associated with steady Lon expression (Kao et al., 2015). We observed Lon and HSP60 protein profiles were similar in NRTI treated HepG2 cells, with increased expression in the acute treatment (Figure 4.1) and significant reduction in the prolonged treatment (Figure 4.2). This further establishes the interaction of Lon and HSP60 in acute stress response.

Both Lon and HSP60 have been reported to be recruited as part of the transmission of ER stress to the mitochondria (Hori et al., 2002). We quantified protein expression of p-eIF2α (Ser51) as an indicator of ER stress. The activation of this protein by phosphorylation results in the repression of translation. The involvement of eIF2α as an ER response to mt damage has previously been reported (Baker et al.,

2012). We observed that p-JNK (an activator of p-eIF2α) and p-eIF2α were significantly elevated during acute exposure to NRTIs (Figure 4.3). This indicates an elevation in misfolded proteins, initiating the ER stress response and induction of Lon expression. Aside from the translational repression elicited by eIF2α, activated JNK regulates co-translational degradation (Gandin et al., 2010). While these responses may be beneficial during acute toxicity, chronic stress requires the initiation of repair and antioxidant responses (Nagiah et al., 2015). Therefore, translational repression cannot be sustained during chronic oxidative stress induced by NRTIs. This is observed in the significant decline in both protein levels at 120 h in all treated HepG2 samples (Figure 4.4). This down-regulation of ER stress response proteins also reduces stress transmission between the ER and mitochondria, reducing Lon and HSP60 activity.

We observed SIRT3 protein expression was elevated by NRTIs in HepG2 cells at both time periods (Figure 4.1, 4.2). SIRT3 is NAD^+ dependent, thus the depletion of ATP associated with sustained mt toxicity does not impact as severely on SIRT3 expression. Loss of SIRT3 expression is associated with aging (McDonnell et al., 2015) and metabolic disorders (Hirschey et al., 2010). The role of SIRT3 in mt metabolism, mt biogenesis (Kong et al., 2010), antioxidant defence (Qiu et al., 2010) and UPR^{mt} (Papa and Germain, 2014) make this protein extremely relevant in mt disorders. The protective role of SIRT3 in mt integrity during chronic stress has previously been reported (Kim et al., 2010). Due to the multiple protective pathways that SIRT3 regulates, it can be expected that this protein is involved in stress responses to NRTI toxicity. Our previous work reported increased protein expression of downstream targets of SIRT3 involved in mt biogenesis (PGC-1α) and antioxidant (SOD1) in the same model (Nagiah et al., 2015). This indicates that SIRT3 provides protection against acute and chronic oxidative stress in NRTI treated hepatocytes.

In conclusion, Lon mediated UPR^{mt} is only relevant to acute toxicity but cannot be sustained due to high energy demand. SIRT3, on the other hand, regulates mt biogenesis, antioxidant defence and UPR^{mt}. Also SIRT3 as a key component in chronic stress response to NRTI induced mt toxicity and oxidative stress, particularly in the AZT and d4T treated cells. Lon and HSP60 protein expression profiles are similar in NRTI induced stress response, possibly due to their functional interaction.

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CHAPTER FIVE

Inverse association between microRNA-124a and *ABCC4* in HepG2 cells treated with antiretroviral drugs

Savania Nagiah [a], Alisa Phulukdaree [ab], Anil Chuturgoon [a]

[a] *Discipline of Medical Biochemistry, School of Laboratory Medicine and Medical Science, College of Health Science, University of KwaZulu Natal, Durban, 4041, South Africa*

[b] *Department of Physiology, School of Medicine, Prinshof Campus, Pretoria, Dr Savage Road, 0083 Prinshof, Pretoria, Gauteng, South Africa* (Current address)

Corresponding author: Prof Anil Chuturgoon, 3rd Floor George Campbell Building, Discipline of Medical Biochemistry, Howard College, University of KwaZulu Natal, King George Avenue, Durban, 4041, South Africa; **email: CHUTUR@ukzn.ac.za**

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Abstract

The ATP-binding cassette (ABC) super-family of drug transporters regulates efflux of xenobiotic compounds. The subfamily, multi-drug resistance proteins (MRPs) transports cyclic nucleotides and xenobiotics. Epigenetic modulation of drug transporters is scarcely described. The regulatory role of microRNA (miR)-124a on drug transporter gene *ABCC4* was only recently reported. Our study investigated the differential regulation of miR-124a by nucleoside reverse transcriptase inhibitors (NRTIs): Zidovudine (AZT), Stavudine (d4T) and Tenofovir (TFV); at 24 h and 120 h treatments in HepG2 cells. *ABCC4* mRNA (qPCR) and ABCC4 protein (western blot) were quantified. Cytotoxicity was evaluated by lactate dehydrogenase (LDH) levels. All NRTIs elevated miR-124a levels at 24 h, with a concomitant decline in *ABCC4* mRNA levels ($p<0.05$). At 120 h, d4T and TFV elevated miR-124a and depleted *ABCC4* mRNA levels ($p<0.0001$), while the inverse was observed with AZT ($p<0.005$). ABCC4 protein was increased by d4T and TFV at 24h. A significant reduction in protein levels was observed at 120 h in all three treatments ($p<0.005$). The disjoint in mRNA and protein levels is likely due to ABCC4 being a membrane bound protein. Following prolonged exposure, membrane integrity was compromised as evidenced by increased LDH leakage ($p<0.005$). We conclude antiretroviral drugs have varying effects on miR-124a and ABCC4.

Introduction

The balance between drug toxicity and drug resistance depends on the absorption, distribution, metabolism and excretion (ADME) of xenobiotics. This is determined by the activity of drug metabolising enzymes and cellular drug transporters. Variability of ADME in different organs will either result in enhanced or limited drug exposure. Past studies on drug toxicology focussed on the metabolism of drugs (phase I and II) and consequent production of free radicals. However, phase III, which involves the energy dependent export of both non-metabolized and metabolized xenobiotics, has recently gained attention for its role in both drug resistance and cellular detoxification (Xu, et. al. 2005; Doring and Petzinger, 2014).

Cellular detoxification at drug deposition organs, such as the liver and kidney, is mediated by complex drug transporter systems. The super-family of ATP-binding cassette (ABC) drug transporters are responsible for the energy dependent efflux of endogenous and xenobiotic compounds. The ABC family of genes is divided into seven families (*ABCA, ABCB, ABCC, ABCD, ABCE, ABCF, ABCG*) with each sub-group exhibiting varying tissue and substrate specificity (Russell, et al. 2008). The *ABCC* class of genes code for membrane bound multi-drug resistance proteins. Five isoforms of ABCC (1-5) proteins are clinically relevant in the development of adverse drug reactions and drug resistance. The spectrum of ABCC substrates is diverse, including prostaglandins (Reid et al., 2003), bile salts and conjugates, steroids (Zelcer et al., 2003), reduced glutathione (Rius et al., 2003), cyclic nucleotides (Wielinga et al., 2003), antiviral drugs:- Tenofovir (TFV) and adefovir (Imaoka et al., 2007), antihypertensive drugs:- furosemide and hydrochlorothiazide (Hasegawa et al., 2007), and anticancer drugs:- camptothecin (Tian et al., 2005), methotrexate (Chen et al., 2002), and topotecan (Tian et al., 2006). The unique ability of ABCC proteins, particularly ABCC4, to transport both cyclic nucleotides and antiretroviral drugs is of importance for the efficacy and toxicity of nucleoside analogues used in anti-HIV treatment.

The functionality and expression of ABCC4 is highly variable. Genetic polymorphisms in the *ABCC4* gene and stress activators may play a role in the expression of ABCC4 protein, but this has not been consistent across different human populations (Abla et al., 2008, Chai et al., 2011, Xu et al., 2010, Maher et al., 2007, Mentzel et al., 2015). Epigenetic modifications scarcely describe a mechanism in the modulation of cellular drug transport (Ivanov et al., 2014). This form of gene regulation refers to heritable changes in gene expression independent of modifications to the DNA sequence or genotype variation. Mechanisms of epigenetic gene regulation include DNA methylation, histone and chromatin modification, and microRNAs (miRNA).

MiRNAs are small non-coding RNA molecules (18-25 nucleotides) involved in post translational gene silencing. The negative regulation of gene expression is achieved by inclusion of miRNA in an RNA-induced silencing complex (RISC) via complementary base pairing to target genes.

Incorporation into RISC results in either destabilisation of the mRNA by shortening of the poly A tail, strand cleavage, or translational repression (Markova and Kroetz, 2014). MiRNAs have multiple targets, including genes involved in drug metabolism and excretion (Yu, 2009).

Changes to miRNA levels in the presence of antiretroviral drugs will have implications for ABCC4 targets, thus influencing bio-accumulation and toxicity. There is limited information on the differential regulation of miRNA by antiretroviral drugs and subsequently, pharmacokinetic changes. A recent study reported direct regulation of *ABCC4* by miR-124a in human embryonic kidney cells (Markova and Kroetz, 2014). The mechanism of *ABCC4* modulation, however, has not been investigated in liver cells, or in relation to antiretroviral drug exposure. Although miR-124a is predominantly expressed in blood, brain and renal cells, this miRNA has recently been shown to be expressed in pig liver (Mentzel et al., 2015) The regulatory role of miR-124a in the liver (HepG2 cells) was further reiterated in an *in vitro* study by Phulukdaree et. al (2015). The study showed inhibition of miR-124a caused an increase in the mRNA and protein expression of miR-124a target *GAMT* (Phulukdaree et al., 2015). Clearly, there is an emerging role for miR-124a in hepatic stress response.

We evaluated the effect of three antiretroviral drugs of the nucleoside reverse transcriptase inhibitor (NRTI) class on *ABCC4* and ABCC4 expression in human hepatoma (HepG2) cells. These drugs are functional analogues of native nucleotides but lack the 3'- hydroxyl group on the sugar moiety. Chronic exposure to these drugs have been associated with changes to metabolism, mitochondrial dysfunction and hepatic steatosis (Brinkman et al., 1998, Begriche et al., 2011). In the present study we investigated differential regulation of miR-124a induced by NRTIs in two time periods (24 h; 120 h) and the subsequent effect on *ABCC4* mRNA levels and ABCC4 protein expression.

Methodology

Materials

All materials were purchased from Merck (Darmstadt, Germany) unless otherwise stated. Antibodies used in western blot protein detection were purchased from Cell Signalling Technology Inc (Beverly, MA, USA). Cell culture medium was obtained from Lonza BioWhittaker (Johannesburg, South Africa) and supplements were obtained from Sigma-Aldrich (St Louis, MO, USA). Antiretroviral drugs were donated as part of the NIH AIDS program.

Treatment

HepG2 cells ($1.5x10^6$) were seeded in $25cm^3$ sterile cell culture flasks and maintained in cell culture medium (10% foetal calf serum, 1% L-glutamine, 1% penstrepfungizone) at 37°C, 5% CO_2. Once cells had reached 70%-80% confluence, treatments were administered. NRTI stock solutions (10mM)

were made up in dimethyl sulfoxide. Cells were treated with maximum plasma level concentrations of NRTIs (Venhoff et al., 2007, Walker et al., 2002): AZT (7.1µM), d4T (4µM) and TFV (1.2µM); for 24 h and 120 h. For the prolonged treatment, cells were rinsed with 0.1M phosphate saline buffer (PBS) and replenished with fresh media containing the treatment.

Cytotoxicity assay

The lactate dehydrogenase (LDH) cytotoxicity detection kit (Roche, Mannheim, Germany) was used to measure the cell damage and cytotoxicity in NRTI treated HepG2 cells. Extracellular LDH activity was quantified by incubating 100µl of cell culture supernatant with 100µl substrate solution (diaphorase/NAD^+; INT/sodium lactate) in a 96-well microtitre plate at room temperature for 30 minutes. The assay was performed with six technical replicates. Optical density (OD) was measured following incubation at 500nm using a spectrophotometric plate reader (Bio-Tek uQuant, Winooski, VT, USA).

RNA isolation and mi-RNA expression

Following treatment, cells were rinsed with 0.1M PBS. Thereafter, 500µl of QIAzol Lysis Reagent (Qiagen, Maryland, USA) were added to each cell culture flask. Total RNA was isolated by an in house protocol based on isopropanol precipitation as described by Chuturgoon et al. (Chuturgoon et al., 2014). Total RNA was quantified using the Nanodrop 2000 spectrophotometer (ThermoScientific, South Africa) and standardized to 2000ng/µl in nuclease free water. Standardized RNA samples were then converted to complementary DNA (cDNA) using the miScript II RT kit (Qiagen, Maryland, USA). MiR-124a expression was analysed using the miScript SYBR Green PCR kit (Qiagen, Catalog no. 218073) according to the manufacturer's instructions. An internal control (RNU6B), was used as a reference control gene to normalize miR-124a expression. .

ABCC4 mRNA expression

Isolated RNA was converted to cDNA by the iScript™ cDNA Synthesis kit (BioRad; catalog no. 107-8890) as per the manufacturer's instructions. A reaction volume of 20µl containing 5X iScript reaction mix, 1µl of iScript reverse transcriptase and 2,000ng of RNA template were made up in nuclease free water. Thermocycler conditions for cDNA synthesis were 25°C for 5 min, 42°C for 30 min, 85°C for 5 min and a final hold at 4°C. The levels of *ABCC4* mRNA were quantified using the iQ™ SYBR® Green Supermix (BioRad, 170-880). A reaction volume of 25µl consisting of 1µM of sense primer (5'-CGATGAGCCATGTGCCATATGA-3'), 1µM antisense primer (5'-TTGACTATCTATCTGGCCTGTGGTTGT-3'), 5X iScript reaction mix and nuclease free water were subject to thermocycler conditions. Initial denaturation was conducted at 95°C for 4 min, followed by 37 cycles of denaturation (95°C; 15 sec), annealing (58°C ; 40 sec), and extension (72°C; 30 sec). An internal control (GAPDH) was also run simultaneously.

All qPCR experiments were conducted on the CFX96 Touch^{TM} Real-Time PCR Detection System (BioRad, Hercules, CA, USA). Changes to gene expression were calculated according to the methods described by Livak and Schmittgen (Livak, 2001). Results are converted from mean relative fold change ($2^{-\Delta\Delta Ct}$) to percentage mRNA expression relative to the untreated control (100%).

Relative protein quantification of ABCC4

Relative quantification of ABCC4 protein was done using western blotting. Crude protein was extracted from HepG2 cells following treatment with NRTIs. Cells were rinsed twice with 0.1M PBS. Thereafter, 200µl of Cytobuster (Novagen, San Diego, CA, USA) supplemented with protease and phosphotase inhibitors (Roche, Mannheim, Germany), was added to cell culture flasks. Flasks were placed on ice (30 min) prior to cells being scraped and transferred to 1.5ml sterile tubes. Lysates were centrifuged (13,000 *g*, 10 min), allowing aspiration of the crude protein supernatant. Crude protein quantification was carried out via the bicinchoninic assay. Protein samples were standardized to 1mg/ml and boiled in Laemmeli buffer [dH_2O, 0.5M Tris-HCl (pH 6.8), glycerol, 10% SDS, β-mercaptoethanol, 1% bromophenol blue] for 5 min.

SDS polyacrylamide gel electrophoresis (4% stacking gel, 10% resolving gel; 1 h; 150 V) was applied to separate protein samples according to molecular weight. The separated proteins were blotted on to nitrocellulose using the TransBlot Turbo Transfer System (BioRad, Hercules, CA, USA) at 400mA for 45 min. Following transfer, the nitrocellulose membrane was incubated in blocking solution [5% non-fat dry milk in Tween 20-Tris buffered saline (TTBS)] for 1 h on a shaker. The membrane was probed with rabbit anti-ABCC4 of 1:500 dilution (cat no. D1Z3W, Cell Signalling Technology, Beverly, MA, USA) for 1 h at room temperature, prior to overnight incubation at 4°C. Following probing with primary antibody, membranes were rinsed 5 times (10 min) in TTBS. Membranes were incubated in HRP-conjugated goat anti-rabbit (ab6112; 1:5,000 dilution) for 1 h at room temperature. This was followed by 5 rinses with TTBS. Clarity Western ECL Substrate (BioRad, Hercules, CA, USA) detection reagent was used to visualise protein bands. Images were captured using gel documentation system Alliance 2.7 (UviTech, Cambridge, UK). Relative band density (RBI) was measured by densitometric analysis using UviTech Alliance Analysis software (Cambridge, UK).

Membranes were incubated in 5% hydrogen peroxide to quench the chemiluminescent signal. The membranes were then probed with a house keeping protein [β-actin (Sigma, St Louis, MO, USA)/] to normalize the results. Results are reported as percentage change relative to control (100%)

Statistical analysis

Statistical analyses were performed using the GraphPad Prism V5 software package (GraphPad Software Inc., San Diego, CA, USA). All experiments were carried out in triplicate unless otherwise

stated. Comparisons between the control and drug treatments were done by performing one-way analysis of variance (ANOVA). Statistical significance was accepted at p value < 0.05.

Results

MiR-124a levels

Levels of miR-124a were increased in all NRTI treatments at 24 h, with a significant increase in the TFV treated HepG2 cells (Figure 5.1A; $p = 0.0009$). Following prolonged exposure, d4T and TFV sustained elevated miR-124a levels relative to the control (Figure 5.1B; $p < 0.0001$). However, miR-124a levels of the AZT treatment in this time period were reduced to 26.25% of the untreated control ($p = 0.02$).

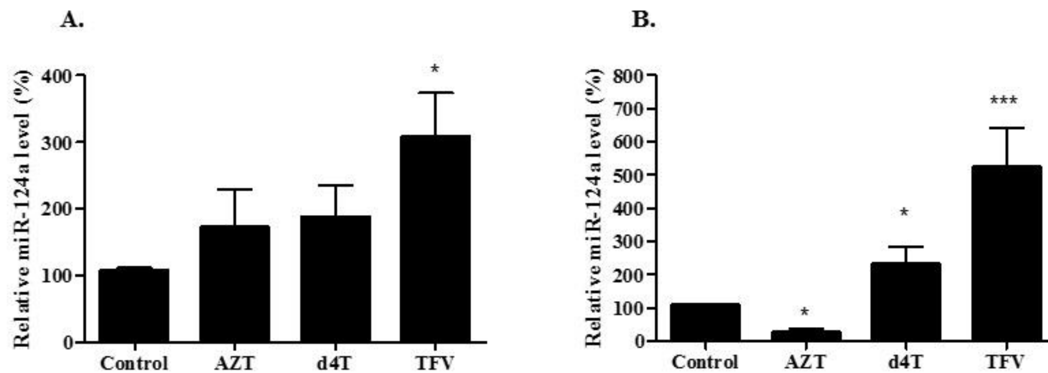

Figure 5.1: Quantitative PCR analysis of miR-124a levels in HepG2 cells following exposure to antiretroviral drugs or 24 h (A) and 120 h (B). MicroRNA-124a levels are expressed in percentage (%) relative to control (100%; *p < 0.05, ***p < 0.001 relative to control)

ABBC4 mRNA levels

MiR-124a results in degradation of target gene *ABCC4* by complementary base pairing, thus lowering *ABCC4* mRNA levels. Evaluation of *ABCC4* mRNA levels in HepG2 cells following acute and prolonged exposure showed that NRTIs caused a significant decline in *ABCC4* mRNA levels at 24 h, with the lowest levels observed in the TFV (9.34%) and d4T (5.59%) treated cells (Figure 5.2A; $p < 0.0001$). The 120 h results showed d4T and TFV treated HepG2 cells had consistently low *ABCC4* mRNA (Figure 2B), which corresponded with the elevated miR-124a levels (Figure 5.1A). The AZT treatment, however, increased *ABCC4* mRNA levels (Figure 5.2B, $p = 0.003$), with a corresponding depletion of miR-124a at 120 h (Figure 5.1B).

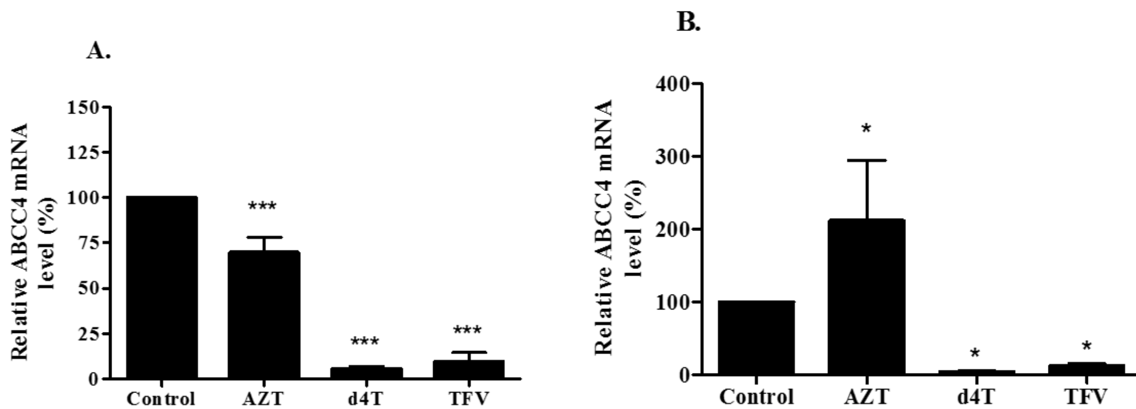

Figure 5.2: Quantitative PCR analysis of ABBC4 mRNA levels at 24 h (A) and 120 h (B) in HepG2 treated with antiretroviral drugs. ABBC4 mRNA is represented as a percentage relative to untreated control (*$p < 0.05$; ***$p < 0.0001$ relative to control)

Relative ABCC4 protein quantification

Densitometric analysis of ABCC4 western blot images showed no change in the AZT treated cells at 24 h. In the same time period, TFV and d4T significantly increased ABCC4 protein expression (Figure 5.3A; $p = 0.005$). At 120 h all three NRTIs caused a significant decline in ABCC4 protein expression (Figure 5.3B; $p < 0.0001$).

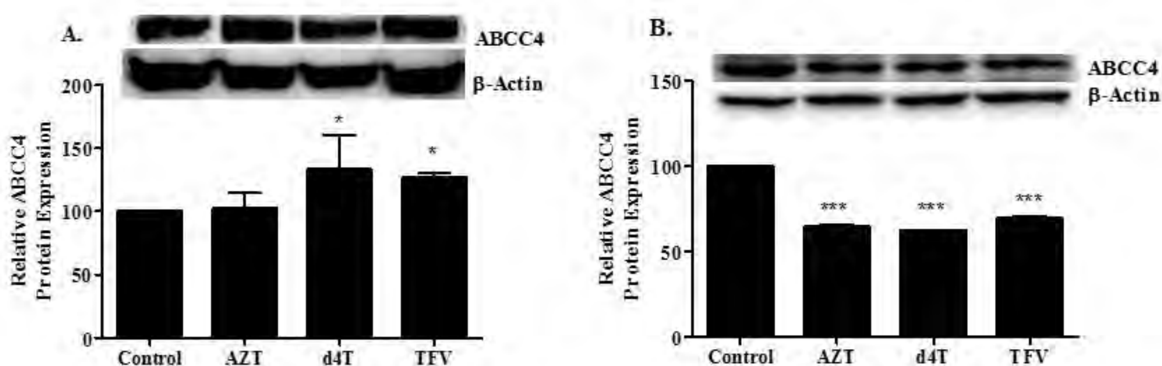

Figure 5.3: Densitometric analysis of western blot for ABCC4 protein at 24 h (A) and 120 h (B) exposure to NRTIs (*$p < 0.05$; ***$p < 0.0001$ relative to control)

Extracellular LDH

Extracellular LDH was quantified as a measure of cytotoxicity. All NRTIs significant increased extracellular LDH activity at both time periods (Figure 5.4; $p < 0.001$).

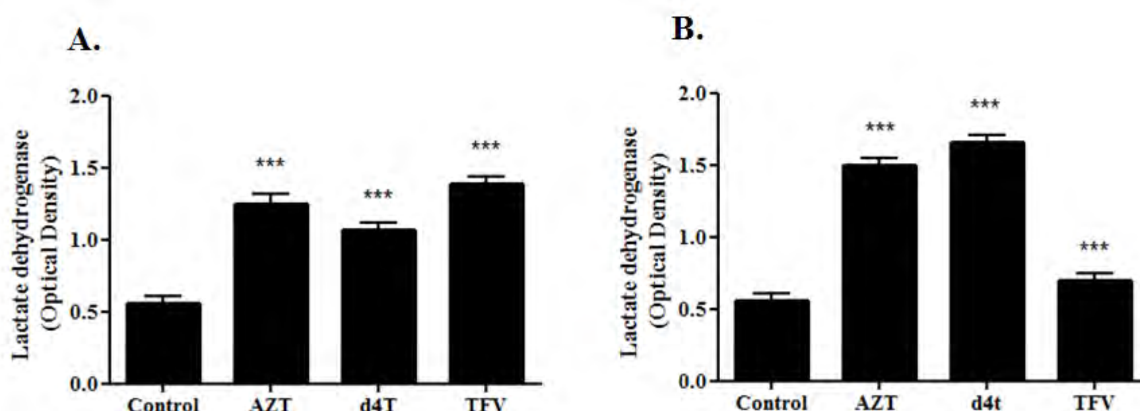

Figure 5.4: Extracellular LDH levels used as an indicator of cytotoxicity was significantly increased in NRTI treated cells at both 24 h (A) and 120 h (B); ******p* **< 0.0001 relative to control**

Discussion

The long term use of antiretroviral therapy has seen the emergence of an "epidemic within an epidemic". Although the morbidity and mortality of HIV infection improved since the advent of antiretroviral therapy, long term side effects (metabolic syndrome, lipodystrophy, hepatic steatosis), have reduced the quality of life of patients (Brinkman et al., 1998). Pharmacokinetic properties of drugs are imperative to understanding mechanisms of drug toxicity. Detoxification pathways in particular have physiological relevance in determining possible adverse drug reactions.

There is limited information on the role of epigenetic mechanisms in drug transport and excretion, especially for chronic drug usage. Recent advancements in this field, however, show miRNA as key components in drug transport (Smith et al., 2012). Borel et. al (2012) showed the involvement of 13 miRNAs that targeted ABC transporters: ABCC1, ABCC5, ABCA1, ABCC10, ABCE1 in hepatocellular carcinoma (Borel et al., 2012). ABCA1 is regulated by miR-33 and miR-758 (Ramirez et al., 2011). Breast cancer resistance protein, ABCG2, is regulated by hsa-miR-328; -519c; -520h (Li et al., 2011). Markova and Kroetz (2012) showed a regulatory mechanism between miR-124a and *ABCC4* in human embryonic kidney cells (Markova and Kroetz, 2014). Using this study, we investigated the effect of miR-124a on *ABCC4* mRNA and ABCC4 protein in HepG2 cells treated with NRTIs. ABCC4 is of significance to NRTI detoxification due to its unique ability to transport both metabolized drugs and cyclic nucleotides (Wielinga et al., 2003). The varying effects of different NRTIs on miR-124a and *ABCC4* is likely to have consequences for hepatic clearance.

Complementary base pairing between the miR-124a seed region and the target sequence of *ABCC4* facilitate binding of RISC to the 3'-UTR of *ABCC4*. This binding results in disruption to the putative binding site on the *ABCC4* gene, causing mRNA degradation (Markova and Kroetz, 2014). Our study showed that miR-124a was elevated by d4T and TFV at both 24 h and 120 h (Figure 5.1), with concomitant repression of *ABCC4* mRNA (Figure 5.2). The inverse relationship between miR-124a and *ABCC4* was further reiterated in AZT treated HepG2 cells, where an increase in miR-124a was accompanied by reduced *ABCC4* mRNA levels at 24 h, while the inverse was applicable at 120 h. The reduced mRNA levels of *ABCC4* did not correlate with ABCC4 protein expression at 24 h in the NRTI treated cells.

ABCC4 regulates cellular detoxification, thereby reducing cytotoxicity. At 24 h, d4T had the lowest LDH extracellular activity relative to AZT and TFV treated cells in the same time period (Figure 5.4A). This treatment also corresponds with the highest ABCC4 protein levels at 24 h (Figure 5.3A). Previously, our investigation on mitochondrial and oxidative stress (using the same experimental model) showed d4T exhibited little or no toxicity at 24 h (Nagiah et al., 2015). The elevated levels of ABCC4 protein may have contributed to lower cytotoxicity as compared to AZT and TFV. At 120 h, however, we observed severe depletion of ABCC4 protein levels in all NRTI treatments (Figure 5.3B). This was corroborated by the elevated levels of LDH activity at 120 h (Figure 5.2B), suggesting increased cell damage. Our previous work on mitochondrial toxicity also showed heightened toxicity, particularly in the d4T and TFV treatments, at 120 h (Nagiah et al., 2015). Although TFV displayed elevated LDH levels at 120 h compared to the control, it appears the cytotoxicity begins to decline compared to the acute treatment. Reasoning for this observation may be due to TFV eliciting a high antioxidant response at chronic exposure and maintaining mitochondrial DNA levels, promoting cell functionality (Nagiah et al., 2015).

The reduced capacity for cellular clearance of drugs and their metabolites may contribute to cytotoxic events. Over-expression of ABCC4 has been shown to confer resistance to acetaminophen induced hepatotoxicity (Aleksunes et al., 2008). Another study showed ABCC4 suppression caused an elevation in intracellular levels of bio-transformed AZT and the antiretroviral agent lamivudine (Anderson et al., 2006). We observed a decrease in ABCC4 protein expression at 120 h in NRTI treated cells, which reduces the detoxification capacity of the cell. The significant decline in ABCC4 protein levels at 120 h can be due to loss of cellular membrane integrity. Our previous work showed significant elevation of extracellular malondialdehyde, a by-product of lipid peroxidation, following prolonged exposure to NRTIs (Nagiah et al., 2015). Reactive oxygen species are produced during drug metabolism, and are capable of reacting with cell membranes, generating lipid peroxides (Bort et al., 1999). The exacerbated oxidative stress in this time period would result in the progressive decline in cell membrane integrity, reducing the levels of membrane bound protein ABCC4. .

Other possible reasons for the discrepancy in *ABCC4* mRNA levels and ABCC4 protein could be reduced ATP availability and compromised protein assembly. Previously we showed that ATP levels were unaffected at 24 h by d4T and a slight decline in the AZT and TFV treated cells, however, at 120 h there was severe depletion of ATP by all three drugs due to NRTI mitochondrial toxicity (Nagiah et al., 2015). ABCC4 is an ATP-dependent protein and depleted levels of ATP will reduce its activity. Furthermore, the prolonged exposure to NRTIs exacerbates oxidative stress. Oxidative stress causes an accumulation in misfolded proteins and compromises post translational processing of proteins in the endoplasmic reticulum (Phulukdaree et al., 2015). Thus *ABCC4* mRNA is present, but the machinery for processing functional ABCC4 protein may be compromised due to the unfavourable oxidative environment.

Translational regulation of ABCC4 has been previously described (Xu et al., 2010). Activator of ABCC4 protein expression, nuclear erythroid factor 2 related factor 2 (Nrf2) was recently evaluated in our laboratory in the same model. This transcription factor was significantly increased by all three NRTIs at 120 h. In spite of elevation of this ABCC4 activator, protein levels were still significantly lower in this time period. Hence, we can deduce that cell membrane integrity and ATP availability over-ride translational regulation of ABBC4 under prolonged oxidative stress (Nagiah et al., 2015).

In conclusion, the antiretroviral drugs AZT, d4T and TFV differentially regulates miR-124a and influences *ABCC4* gene expression. Prolonged exposure to NRTIs compromises drug export by reducing ABCC4 protein expression, thus contributing to reduced cellular clearance and increased drug toxicity. MiR-124a may influence hepatic clearance of d4T and TFV, thereby reducing hepatic toxicity. Although miR-124a directly regulates *ABCC4* mRNA expression, ABCC4 protein expression is dependent on other factors such as ATP availability and oxidative status of the cell.

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Declaration of Interest

None

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CHAPTER SIX

CONCLUSION

Metabolic syndrome, lipodystrophy, and liver toxicity are common outcomes with long term use of HAART. Nucleoside reverse transcriptase inhibitors, the cornerstone of HAART, are known inducers of mt toxicity. The mechanism by which NRTIs exert mt toxicity is still debatable as the depletion of mtDNA is not consistent in all NRTIs. Furthermore, NRTIs display tissue specific toxicity: d4T – hepatic, adipose (Saint-Marc, Partisani et al. 1999, Setzer, Lebrecht et al. 2008); AZT – hepatic, cardiac, peripheral muscle (Barile, Valenti et al. 1997, Benbrik, Chariot et al. 1997, de la Asunción, del Olmo et al. 2004); TFV – renal (Abraham, Ramamoorthy et al. 2013).

The liver is of particular importance in relation to drug toxicity as it is central to xenobiotic metabolism. The liver is at high risk for both oxidative insult due to drug metabolism and drug deposition. Mitochondrial toxicity and oxidative stress are common in NRTI toxicity with each NRTI having differing degrees of toxicity. Understanding the molecular aetiology of NRTI induced liver toxicity may aid in the clinical management of HIV-therapy. We selected three of the most commonly used NRTIs (AZT, d4T, TFV) used in fixed dose combinations in developing countries (Simbayi, Shisana et al. 2014). This *in vitro* study was designed to represent both acute and prolonged exposure to NRTIs in order to evaluate changes in stress response profiles.

Both AZT and TFV compromised ATP production and elevated biomarkers for oxidative stress at 24 h. Stavudine only induced oxidative stress and mt dysfunction following prolonged exposure, surpassing the toxicity of AZT and TFV at 120 h. This was accompanied by a significant reduction in mtDNA content (36.1%). Tenofovir was also toxic to mt as observed by both reduced ATP levels and mt membrane polarisation, but this was independent of mtDNA depletion. These findings show that although mtDNA depletion may exacerbate the mt toxicity observed with d4T and AZT, this was not the mechanism of NRTI-associated mt toxicity.

In the assessment of the endogenous HepG2 antioxidant response, TFV induced the highest Nrf2 and SOD1 expression (>2 fold), as well as the DNA repair mechanism, OGG1. Although TFV disrupted cellular energy status, the heightened induction of Nrf2 mediated antioxidant response may explain why TFV is considered less toxic than d4T and AZT in a clinical setting. Further, Nrf2 and its downstream target, SOD1, were markedly elevated after prolonged exposure to NRTIs and strongly suggests that Nrf2 is required for chronic stress response to NRTIs.

Another important stress response to mt toxicity is the regulator of mt biogenesis, PGC-1α; PGC-1α was significantly increased following acute and prolonged exposure to NRTIs in HepG2 cells. Besides its role in mt biogenesis, PGC-1α also induces a battery of antioxidant responses in the

mitochondrion to mt toxicity. The transcription factor -TFAM, responsible for transcribing mtDNA, was only increased after prolonged exposure to NRTIs, indicating this may be a late response.

The NRTI depletion of ATP in HepG2 cells at 120 h reduced ATP-dependent stress responses that regulate mt protein homeostasis. As a result, Lon protease and the associated protein maintenance proteins were elevated after acute NRTI exposure, but was not sustained over prolonged exposure. The dysfunction of Lon due to chronic oxidative stress is associated with age-associated disorders, a characteristic of chronic HAART use. The loss of Lon activity and the translational repressor, eIF2α, seems to promote mt biogenesis and mt antioxidant response. Lon selectively degrades TFAM, and during prolonged stress this may be detrimental to the cell. In order for mt biogenesis to be increased to compensate for mt toxicity, Lon activity needs to be reduced. Furthermore, translational repression by eIF2α will be problematic following prolonged exposure to NRTIs, as the processing of proteins involved in antioxidant response will need to be increased. Post-translational regulation of Lon by SIRT3 may be relevant, as an inverse relationship between these two mt matrix proteins in both time periods was observed. Sirtuin 3 is NAD+ dependent, and depleted ATP levels will not significantly impact on its functionality. Changes in energy and redox status induce SIRT3 activity, which down-regulates Lon and increases mt antioxidant response.

The ATP-binding cassette C4 (*ABCC4*) gene is responsible for the transport of xenobiotic molecules and endogenous nucleotides, making this membrane protein highly relevant for NRTI transport. The activity of this efflux protein determines intracellular drug concentration and facilitates cellular detoxification in the liver. The regulation of cellular drug transporters by miRNA is a potential target for enhancing hepatic drug clearance. The *ABCC4* gene is directly regulated by miR-124a; a mechanism as yet to be described in relation to NRTI hepatic toxicity. The acute exposure to all three NRTIs significantly increased miR-124a levels (24h), with a concomitant decline in *ABCC4* mRNA levels. A similar trend was observed following 120h exposure to d4T and TFV. After 120h exposure to AZT, miR-124a levels significantly decreased, while *ABCC4* mRNA levels increased. Although a negative association between miR-124a and *ABCC4* became apparent, this could not be correlated with protein expression of the *ABCC4* gene (MRP4). A significant decrease in MRP4 was observed in all treatments at 120h, compromising the export of the NRTIs. Since MRP4 is a membrane bound protein, the loss of cell membrane integrity due to lipid peroxidation (as observed by elevated MDA content) can negatively impact protein expression. Furthermore, MRP4 requires ATP for its efflux activity and the decreased ATP levels (120h) to NRTIs will reduce MRP4 capacity.

In conclusion the data shows that mt function and oxidative stress are induced by AZT, d4T and TFV, especially at 120h, in HepG2 cells. The consequent disruption of energy production has implications for long term stress responses, including those involved in protein maintenance and cellular detoxification. MicroRNA that target cellular detoxification proteins provide a potential therapeutic

intervention for NRTI-associated hepatic toxicity. Future studies still need to be done to validate the findings with use of inhibitors/mimics of the key regulators identified (Nrf2, Lon, miR-124a).

Although this study offers insight to molecular events in the presence of acute and prolonged NRTI exposure in liver cells, there are limitations to the interpretation of the results reported. An *in vitro* model always poses the challenge of a unicellular model, thus not accounting for a systemic interactions in a multi-organ environment. Findings from this study, however, provide stepping stones to identifying molecular targets at the clinical level. The pathways investigated can be adapted to *an in vivo* model (animal study) to further refine molecular targets of NRTI toxicity. These findings can then be further investigated in patients exhibiting NRTI-associated adverse health outcomes such as lipodystophy, hepatic steatosis and metabolic syndrome.

APPENDIX A

Apoptosis assessment (Caspase activity)

In order to assess the induction of apoptosis (programmed cell death) in HepG2 cells following acute (24 h) and prolonged (120 h), caspase activity (8/9/3/7) was assessed using a luminometric assay. The Caspase-Glo® 8 Assay, Caspase-Glo® 9 Assay, Caspase-Glo® 3/7 Assay (Promega, Madison, USA) were conducted according to the manufacturer's instructions. Briefly 20 000 cells (50µl in 0.1MPBS) were seeded in a 96-well white microtitre plate with 20µl reagent per replicate. Samples were mixed by agitation and incubated in the dark (RT; 30 min). Luminescent signal was detected on a Modulus™ Microplate Luminometer (Turner Biosystems, Sunnyvale, USA). Results are expressed as relative light units (RLU) and converted to fold change relative to the control.

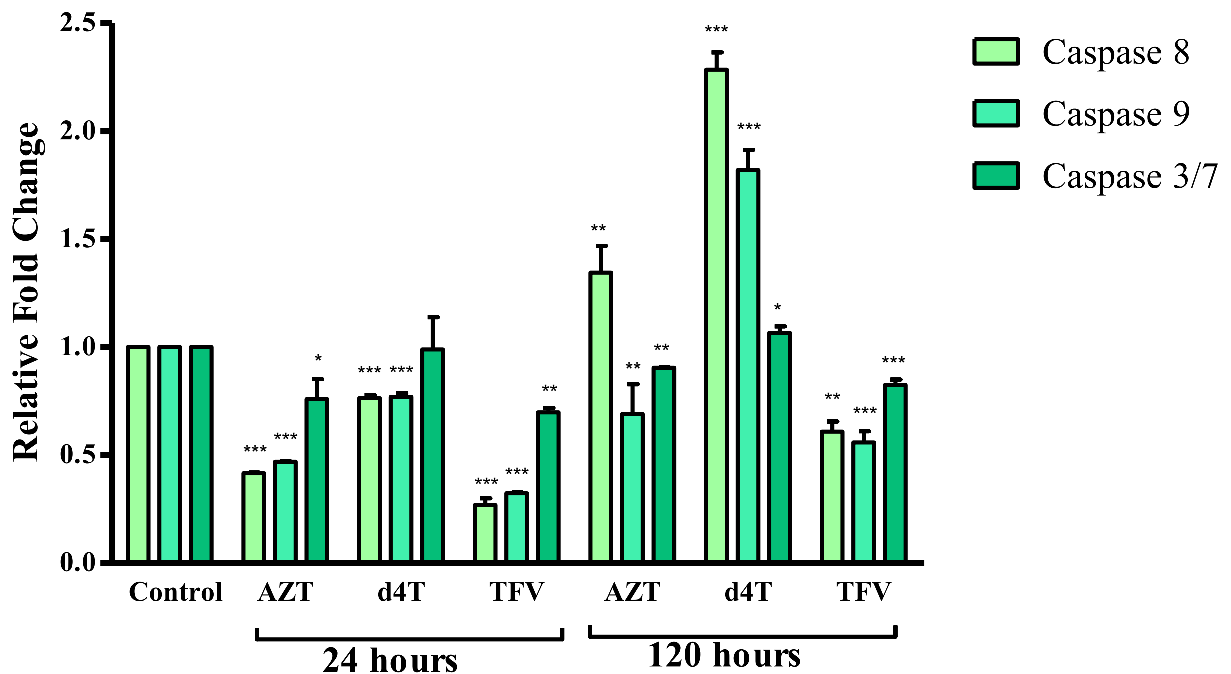

Figure 1: Caspase activity following acute and prolonged exposure to NRTIs; *p < 0.05, **p <0.005, ***p < 0.0001

Early marker for apoptosis, phosphatidyl-serine externalisation, was quantified by Annexin-V-Fluos staining and FACS. Briefly, 1 000 000 cells per treatment were reconstituted in 100µl 0.1MPBS and stained with 100µl of staining buffer, 100µl of Annexin-V-Fluos labelling solution (Annexin-V: propidium iodide (PI): staining buffer (1:1:50 vol/vol/vol)). Cells were incubated in staining solution in the dark (15 min; RT). Flow cytometry data from stained cells (50 000 events) was captured with

the Accuri™ C6 flow cytometer and software (BD Biosciences, San Jose, CA, USA). Live cells were gated using CFlow Plus Software (BD Biosciences, San Jose, CA, USA). Data is represented as percentage apoptotic cells. The results show only TFV elevated phosphatidyl-serine externalisation following acute exposure (Addendum A - Figure 2). At 120 h all NRTIs increased this early marker for apoptosis.

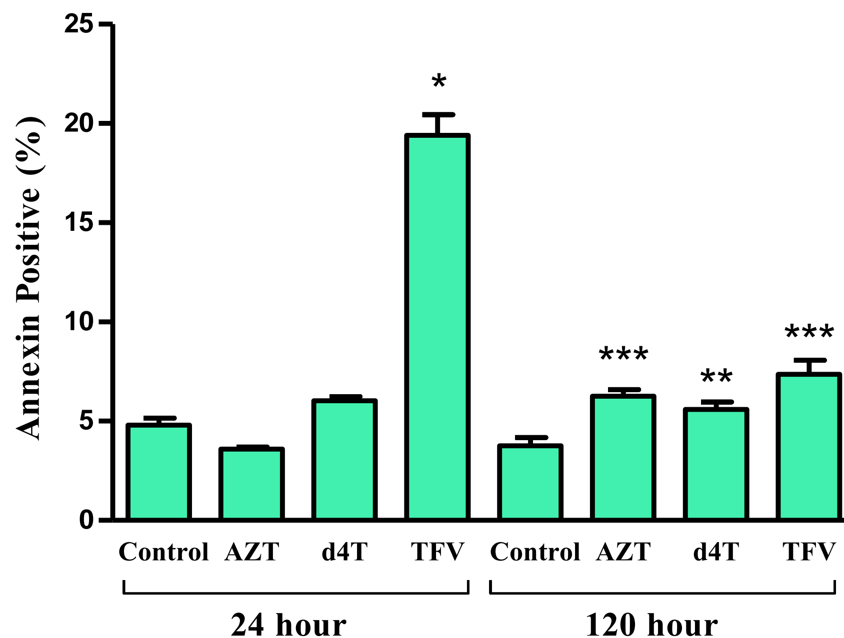

Figure 2: Annexin-V-Fluos data for phosphatidyl-serine externalisation in HepG2 cells treated with NRTIs; *p < 0.05, **p <0.005, ***p < 0.0001

APPENDIX B

LonP1 gene expression

LonP1 gene expression was quantified by qPCR. Isolated RNA was standardized to 1000ng/µl and converted to cDNA using the RT^2 First Strand Kit as per the manufacturer's instructions (Qiagen, Maryland, USA). A predesigned LonP1 Taqman® Gene Expression Assay (ThermoFischer Scientific, Waltham, MA, USA) was used to quantify LonP1 gene expression. The Viia™7 Real Time PCR system was used to conduct thermocycler conditions and capture gene expression data (ThermoFischer Scientific).

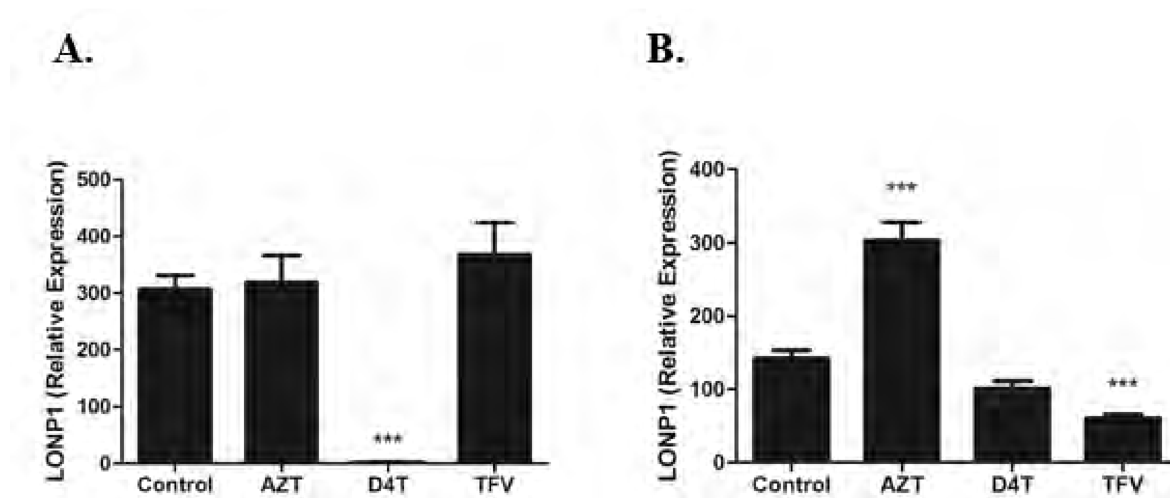

Figure 1: LonP1 gene expression in HepG2 cells following 24 h (A) and 120 h (B) exposure to NRTIs; *p < 0.05, **p <0.005, ***p < 0.0001

APPENDIX C

RNA isolation protocol

The isolation of RNA from HepG2 cells was carried out using an in-house protocol. Following treatment, cells were rinsed thrice with 0.1M PBS. Thereafter, 500µl of QIAzol RNA extraction reagent (Qiagen) and 500µl of 0.1M PBS was added to each flask. Cells were incubated in QIAzol for 5 min at RT. Cells were then mechanically lysed with a cell scraper and lysates were transferred to 1.5ml microcentrifuge tubes and allowed to freeze overnight (-80ºC). Samples were thawed done following incubation and 100µl chloroform was added per sample. Samples were agitated for 15 s and allowed to stand for 3 min at RT. The samples were then centrifuged (15 min; 12 000 x g; 4ºC) and the aqueous phase was transferred to fresh microcentrifuge tubes. Isopropanol (250µl) was added to the aqueous phase and left on ice for 2 h before overnight incubation (-80ºC). Following incubation, samples were centrifuged (20 min; 12 000 x g; 4ºC). The supernatant was discarded and the pellet was rinsed with cold 75% ethanol (500µl). Samples were centrifuged (15 min; 7 400 x g, 4ºC) and the ethanol was discarded. The pellets were allowed to air dry prior to being reconstituted in 15µl of nuclease free water. The reconstituted RNA was allowed to stand at RT for 10 min to allow solubilisation, and then stored at -80ºC.

APPENDIX D

Protein expression (western blot)

The following western blots were run to evaluate protein expression of p-SIRT1 (Addendum D-Figure 1), p-p53 (Addendum D – Figure 2), and SIRT5 (Addendum D –Figure 3) as pro-survival proteins during stress response. Sirtuin 1 is a cytosolic SIRT that has been implicated in biological aging. Both p53 and SIRT1 have been identified for interaction with Lon during cellular senescence. The transcription factor p53 is involved in cell death response and DNA damage response. Sirtuin 5 is located in the mitochondrion and plays a role in metabolism.

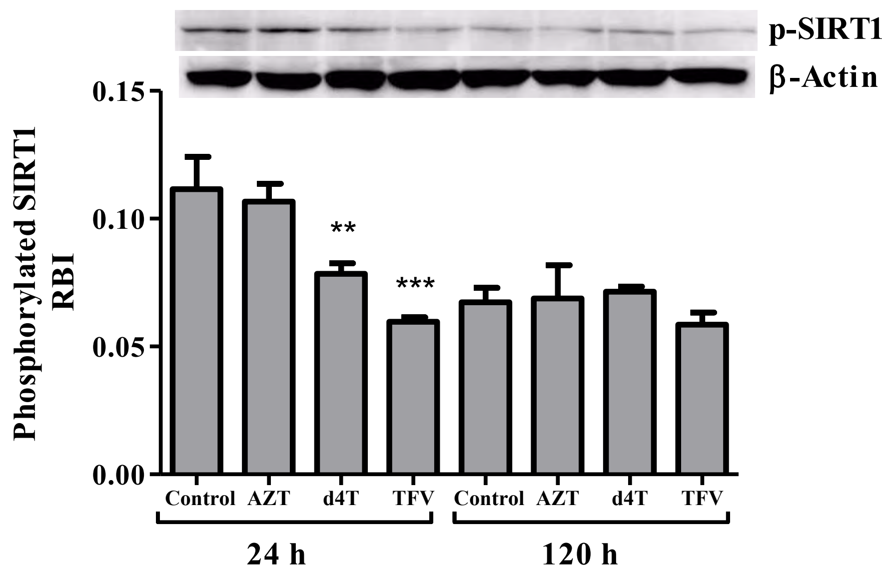

Figure 1: Densitometric analysis of phosphorylated SIRT1 protein expression in HepG2 cells. Data is reported as relative band intensity (RBI) normalized against β-Actin; *$p < 0.05$, **$p < 0.005$, ***$p < 0.0001$

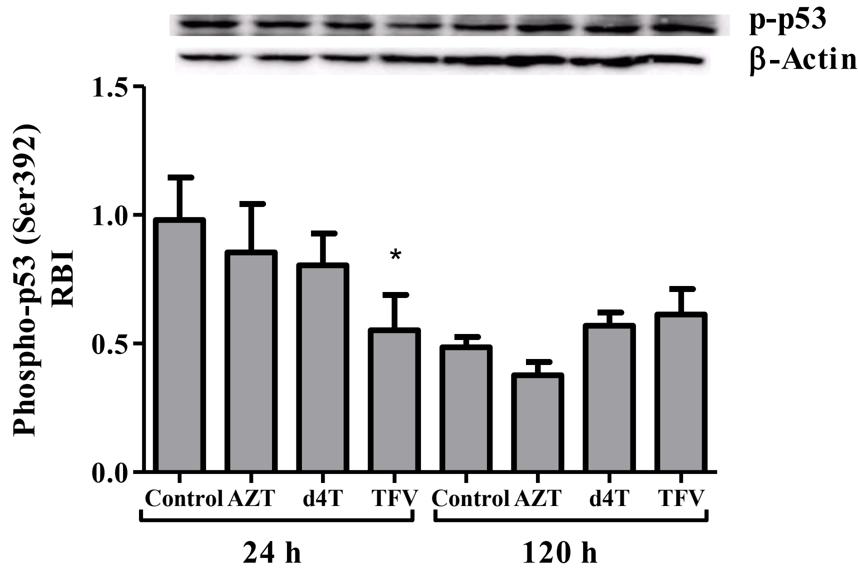

Figure 2: Densitometric analysis of phosphorylated p53 (Ser392) protein expression in HepG2 cells. Data is reported as relative band intensity (RBI) normalized against β-Actin; *p < 0.05, **p <0.005, ***p < 0.0001

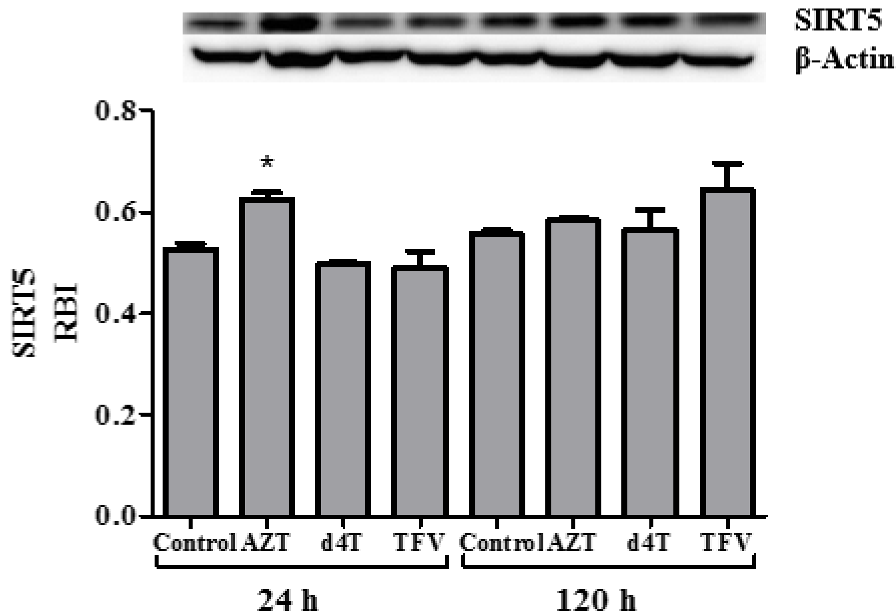

Figure 3: Densitometric analysis of SIRT5 protein expression in HepG2 cells. Data is reported as relative band intensity (RBI) normalized against β-Actin; *p < 0.05, **p <0.005, ***p < 0.0001

APPENDIX E

Intracellular ROS quantification by DCF staining

Intracellular ROS was determined by flow cytometric analysis of DCF positively stained cells. At 120h, all three NRTI treated cells had a higher percentage of DCF positive stained cells relative to the control (Addendum E - Figure 1).

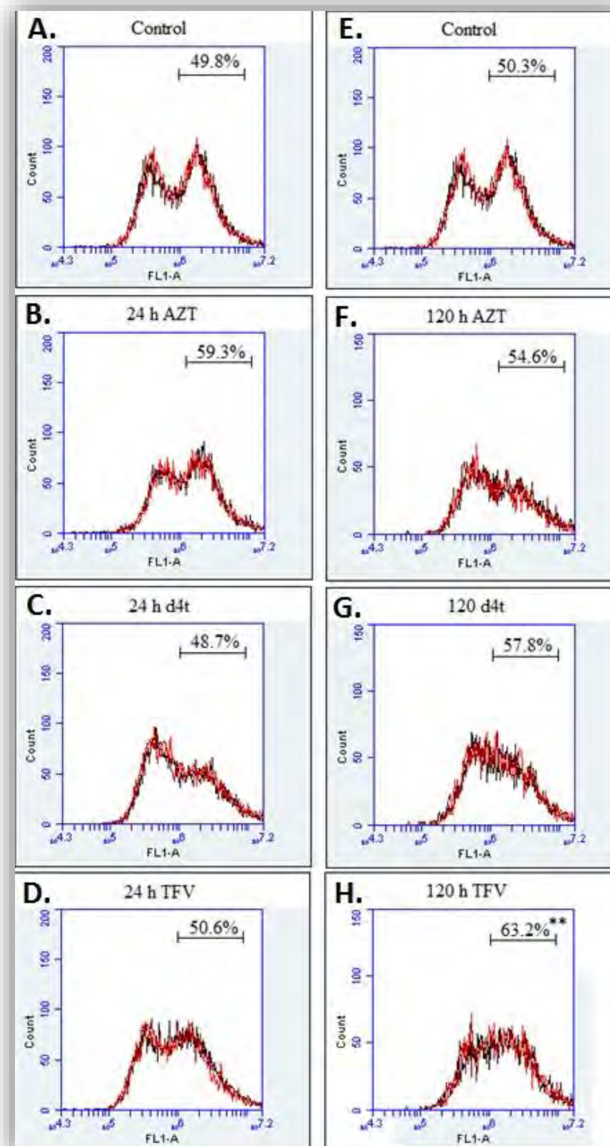

Figure 1: FACS analysis of DCF positive stained cells. At 24 h AZT (B) and TFV (D) had increased levels of intracellular ROS relative to the control (A). At 120 h, however, all three NRTIs had a higher percentage of DCF positive cells compared to the control (E), with TFV exhibiting the highest ROS levels (H)