Potential hepatoprotective effects of naringenin in Nucleoside Reverse Transcriptase Inhibitor-induced oxidative stress and apoptosis in hepatocytes in vitro

KHMERA GOVENDER

212505286

Potential hepatoprotective effects of naringenin in Nucleoside Reverse Transcriptase Inhibitor-induced oxidative stress and apoptosis in hepatocytes in vitro

By

KHMERA GOVENDER

212505286

B.Pharm (UKZN)

Submitted in fulfilment of the requirements for the degree

of

Master of Pharmacy (Pharmacology)

Department of Pharmacology, Discipline of Pharmaceutical Sciences, College of Health Sciences University of Kwazulu-Natal, P.O. Box X5401, Durban, South Africa.

Supervisor: Dr PMO Owira

Date Submitted:

Potential hepatoprotective effects of naringenin in Nucleoside Reverse Transcriptase Inhibitor-induced oxidative stress and apoptosis in hepatocytes in vitro

By

KHMERA GOVENDER

212505286

B.Pharm (UKZN)

Submitted in fulfilment of the requirements for the degree

of

MASTER OF PHARMACY (PHARMACOLOGY)

Department of Pharmacology

Discipline of Pharmaceutical Sciences

College of Health Sciences

University of KwaZulu-Natal

As the candidate's supervisor, I have approved this dissertation for submission

Signed: Name: Date:

PREFACE

The experimental work described in this dissertation was carried out in the Department of Pharmacology, Discipline of Pharmaceutical Sciences, College of Health Science, University of KwaZulu-Natal, Durban from April 2018 to March 2019 under the supervision of Dr PMO Owira.

The study is an original work of the author and has been submitted in fulfilment of the academic requirements for obtaining a Master of Pharmacy Degree in Pharmacology. Information from other sources used in this dissertation has been duly acknowledged in the text and reference section.

Khmera Govender

Dr PMO Owira

DECLARATIONS

DECLARATION: PLAGIARISM

I, **Khmera Govender** declare that:

1. The research reported in this thesis, except where otherwise indicated, is my original work.

2. This thesis has not been submitted for any degree or examination at any other university.

3. This thesis does not contain other persons' data, pictures, graphs or other information, unless specifically acknowledged as being sourced from other persons.

4. This thesis does not contain other persons' writing, unless specifically acknowledged as being sourced from other researchers. Where other written sources have been quoted, then:
a) Their words have been re-written but the general information attributed to them has been referenced.
b) Where their exact words have been used, then their writing has been placed in italics and inside quotation marks, and referenced.

5. This thesis does not contain text, graphics or tables copied and pasted from the internet, unless specifically acknowledged, and the source being detailed in the thesis and in the References sections.

Signed: _____

ACKNOWLEDGEMENTS

This work would not have been possible without the unwavering support of my family. I would like to thank my parents whose love and guidance are with me in whatever I pursue. They are my ultimate role models. I also wish to thank my amazing husband whose love and encouragement has always inspired me to be the best version of myself.

I am grateful to my supervisor Dr Peter Owira for his invaluable guidance and wisdom throughout this project.

My heartfelt thanks to Ms Kogi Moodley and the staff at the UKZN physiology department for their assistance as well as the National Research Foundation (NRF) for financial support.

LIST OF ABBREVIATIONS

ABC	Abacavir
ADP	Adenosine diphosphate
AIDS	Acquired Immunodefiency syndrome
ALAT	Alanine transaminase
APV	Amprenavir
AST	Aspartate aminotransferase
ATP	Adenosine triphosphate
AZT/ZDV	Zidovudine
Bak	BC2-antagonist/killer
Bax	Bcl-2 associated X protein
Bcl-2	B-cell CLL/Lymphoma 2
cART	Combination antiretroviral therapy
CCl_4	Carbon tetrachloride
CCR5	C-C chemokine receptor type 5
CD4	Cluster of differentiation 4
COX-2	Cyclooxygenase-2
CXCR4	C-X-C chemokine receptor type 4
CYP	Cytochrome

d4T	Stavudine
ddC	Zalcitabine
ddL	Didanosine
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DP	Diphosphate
DRV	Darunavir
EFV	Efavirenz
ER	Endoplasmic reticulum
ETC	Electron transport chain
ETR	Etravirine
FADD	Fas-associated protein with a death domain
fAPV	Fosamprenavir
FFA	Free fatty acids
FTC	Emtricitabine
GCL	Glutamyl cysteinyl ligase
GPx	Glutathione peroxidase
GSH	Glutathione
GSSG	Glutathione disulfate
GST	Glutathione S transferase

HAART	Highly Active Antiretroviral Therapy
HIV	Human Immunodeficiency Virus
HIV-RT	HIV reverse transcriptase
HO-1	Heme oxygenase 1
HRP	Avidin-Horseradish Peroxidase
IDV	Indinavir
iNOS	Nitric Oxide Synthase
INH	Isoniazid
LPV	Lopinavir
MAC	Maraviroc
Maf	Musculoaponeurotic fibrosarcoma
MAPK	Mitogen-activated protein kinases
MDA	Malondialdehyde
MP	Monophosphate
mtDNA	Mitochondrial DNA
mtRNA	Mitochondrial ribonucleic acid
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NADPH	Nicotinamide adenine dinucleotide phosphate
NfV	Nelfinavir
NNRTI	Non-Nucleoside Reverse Transcriptase Inhibitors

NQO1	NAD(P)H Quinone Dehydrogenase
Nrf2	Nuclear factor-erythroid 2 related factor
NRTI	Nucleoside Reverse Transcriptase Inhibitors
NVP	Nevirapine
OATP	Organic anion transporting protein
OXPHOS	Oxidative phosphorylation
PBS	Phosphate buffer solution
P-gp	p-glycoprotein
PI	Protease Inhibitors
RAL	Raltegravir
RIF	Rifampicin
ROS	Reactive oxygen species
RPV	Rilpivirine
RTV	Ritonavir
SOD	Super Oxide Dismutase
SQV	Saquinavir
T-20	Enfuvirtide
3TC	Lamivudine
TDF	Tenofovir
TNF	Tumor necrosis factor

TNF-α	Tumor necrosis factor-α
TPV	Tipranavir
TP	Triphosphate
UNAIDS	United Nations Programme on HIV/AIDS
UGT	UDP-glucuronosyl transferase
WHO	World Health Organisation
XO	Xanthine oxidase

LIST OF FIGURES

LIST OF TABLES

PULICATIONS

1. Lutu MR, Nzuza S, Mato PEM, **Govender K**, Mphumelelo Gumede LM, Kumalo SI,Mlambo NN, Hurchund R, Owira PMO. DNA polymerase-γ hypothesis in nucleoside reverse transcriptase-induced mitochondrial toxicity revisited: A potentially protective role for citrus fruit derived naringenin? European Journal of Pharmacology. 2019; 852:159-166.

2. **Govender K**, Mato PEM, Nzuza S, Kometsi L, Hurchand R, Gumede L, Kumalo SI. Potential hepatoprotective effects of naringenin in combination antiretroviral therapy. Journal of Pharmacy and Pharmacology, JPP-19-0358, 24-04-2019, Manuscript has been submitted.

TABLE OF CONTENTS

Contents

ABSTRACT

Introduction: Nucleoside Reverse Transcriptase Inhibitors (NRTIs) are known as the backbone of Combination Antiretroviral Therapy. Metabolic complications such as hyperlactatemia, steatosis, steatohepatitis, hepatomegaly, and abnormal liver functions have been associated with NRTI treatment, which potentially contribute to poor patient compliance and adherence. Studies suggest that NRTIs inhibit mitochondrial DNA pol-γ, generate reactive oxygen species and cause defective oxidative phosphorylation as the underlying mechanism of mitochondrial dysfunction in the liver and other tissues. Oxidative stress induced by NRTIs contributes to and initiates the progression of liver injury. Naringenin, a plant-derived flavanone has demonstrated antioxidant, and anti-apoptotic properties that may be useful in ameliorating NRTI-induced hepatocyte injury.

Aim: To investigate the potential protective effects of naringenin in NRTI-induced oxidative stress and apoptosis in Chang cells.

Methods: Chang cells in culture medium were exposed to steady state plasma concentrations of stavudine (4 μM), zidovudine (7.1 μM) and treated with or without naringenin (10-50 μM) for 24 hours, to determine the antioxidant and anti-apoptotic effects of naringenin on NRTI-exposed cells using Super Oxide Dismutase (SOD) assay, Catalase assay, Glutathione peroxidase (GPx) assay, MTT assay, and Caspase-3 and -9 assay respectively.

Results: Cell viability, SOD, catalase and GPx activities were significantly reduced by AZT and d4T respectively. Treatment with increasing concentrations of naringenin significantly improved cell viability and increased SOD, GPx and catalase activities in cells treated with AZT and d4T. Furthermore, co-treatment with naringenin in AZT and d4T treated cells significantly reduced caspase-3 and caspase-9 activities.

Conclusion: AZT and d4T induce oxidative stress and apoptosis in Chang cells. Treatment with naringenin protected the cells against oxidative damage and consequently prevented apoptosis in a dose-dependent manner. Our results therefore suggest further investigation into being used naringenin as a reliable nutritional supplement that could ameliorate NRTI-induced hepatic injury caused by oxidative stress and apoptosis in HIV patients.

CHAPTER ONE

Introduction

1.1 HIV Epidemiology

According to United Nations Programme on HIV/AIDS (UNAIDS) reports, an estimated 76.1 million people have been infected with Human Immunodeficiency Virus (HIV) since the Acquired Immunodefiency Syndrome (AIDS) epidemic began [1]. Recent statistics show that 36.7 million people are living with HIV infections [1]. Majority of people living with HIV are from low and middle income countries [2]. Of the 36.7 million people infected with HIV, 70% of people newly infected and living with HIV are from sub-Saharan Africa, more so, Eastern, Central and Southern Africa. Women are the most affected, accounting for 59% of the population living with HIV in sub-Saharan Africa, which accounts for 43% of the worldwide total of new HIV infections [1]. South Africa has the highest number of HIV patients despite the relatively low population. Statistics indicate that 20% of South Africa's population are either newly infected or living with HIV [2]. Recent statistics show that there have been 110 000 AIDS related deaths in South Africa alone [3].

The overall number of people living with HIV has increased, not only due to the number of new infections, but this is also attributed to the introduction of more widely obtainable antiretroviral therapy (ART) [2]. The increased availability of combination antiretroviral therapy (cART) has led to a dramatic rise in life expectancy in HIV-infected patients [4], which has averted approximately 5.2 million AIDS-related deaths in low- and middle income countries from 1995 to 2010 [5]. In South Africa, 56% of the population living with HIV are on ART [3].

1.2 Antiretroviral Drugs

Significant developments in the efficacy of and tolerability to antiretroviral (ARV) drugs has reduced plasma viral loads, prevented transmission, and slowed down the progression of HIV infection, thereby enhancing life expectancy and improving quality of life [2, 6]. Although there is no curative treatment available for HIV, the advent of ARV drugs has changed the HIV infection to a chronic and manageable condition, rather than a terminal illness [7]. Currently, there are 6 classes of ARV drugs available namely, Nucleoside/Nucleotide Reverse Transcriptase Inhibitors(NRTIs); Non-Nucleoside Reverse Transcriptase Inhibitors (NNRTIs); Protease Inhibitors (PIs); Fusion Inhibitors (FIs); Integrase Inhibitors and Co-Receptor/Entry Inhibitors [8] (Table 1).

Table 1: Classes of medications currently used to treat HIV infections [6, 9, 10, 11].

Classes of ARVs	Drug name	Mechanism of action
Nucleoside/Nucleotide Reverse Transcriptase Inhibitors(NRTIs)	Abacavir (ABC) Zidovudine Didanosine (ddI) (AZT/ZDV) Emtricitabine (FTC) Lamivudine (3TC) Stavudine (d4T) Tenofovir (TDF) Zalcitabine (ddC)	Counterfeit incorporation mechanism leading to termination of viral cDNA chain elongation via inhibition of reverse transcriptase.
Non-Nucleoside Reverse Transcriptase Inhibitors (NNRTIs)	Efavirenz (EFV) Etravirine (ETR) Nevirapine (NVP) Rilpivirine (RPV)	Inhibit viral reverse transcriptase by allosteric hindrance leading to termination of viral cDNA chain elongation
Protease Inhibitors (PIs)	Amprenavir(APV) Nelfinavir (NfV) Atazanavir(ATV) Ritonavir(RTV-booster) Darunavir (DRV) Fosamprenavir(fAPV) Saquinavir (SQV) Indinavir(IDV) Tipranavir (TPV) Lopinavir (LPV)	Inhibit viral proteases, which cleave viral polyproteins into mature functional proteins hence they block viral maturation.
Fusion Inhibitors (FIs)	Enfuvirtide (T-20)	Inhibits fusion of the viral envelope with CD4 cell's membrane C-region gp41 sequences
Integrase Inhibitors	Raltegravir (RAL)	Block integration of proviral DNA into the CD4 cell chromosomal DNA
Co-Receptor/Entry Inhibitors	Maraviroc (MAC)	Chemokine CCR5 receptor antagonist. Blocks entry of the viral RNA into the CD4 cell.

NRTIs are structural analogues of innate nucleosides which competitively bind to the active site of HIV reverse transcriptase. Hence, NRTIs terminate chain elongation by counterfeit incorporation mechanism [12, 13]. NNRTIs non-competitively bind to the hydrophobic pockets of the reverse transcriptase enzyme and induce conformational changes which reduces its catalytic activity thus preventing replication of the reverse transcriptase enzyme [14, 15]. Protease inhibitors bind to the substrate side of the HIV protease enzyme thus inhibiting the final maturation for the production of mature viral proteins which proceed to infect other cells [16]. Integrase inhibitors block the active site of Mg^{2+} within HIV-1 integrase, thus preventing the active site from binding to target genomic DNA strand. This prevents the integration of viral DNA in the host's nuclear genome [17]. Fusion inhibitors block the fusion of the virus particles on the outer cell membrane by engaging in a coil-coil interaction with regions of viral glycoprotein gp41 [18]. Co-receptor Inhibitors or entry inhibitors bind to CCR5 or CXCR4 receptors. This prevents the interaction of viral gp120 and the CD4 receptor which is required in order to enter target cells [18, 19] (Table 1).

cART involves the use of a combination of ARV classes to sufficiently suppress the viral replication of HIV within the host. The goal of cART is to reduce the plasma viral load to less than 50 HIV RNA copies and increase CD4 counts [14]. Current international guidelines for management of HIV use 3 classes of drugs, which includes two NRTIs combined with one NNRTI or PI or Integrase inhibitor [6, 20, 21]. NRTIs was the first class of ARVs to become approved for the treatment of HIV and remain the backbone of cART [14]. The use of FDC's has improved adherence to therapy, lowered the cost of generic fixed-dose combinations, reduced pill burden and reduced the occurrence of opportunistic infections. The combination of NRTIs prescribed is based on patient tolerability profile as well as risk of

potential side-effects [12]. Several ARVs however, have been removed from therapeutic regimens due to the development of undesirable adverse-effects [6].

1.3 Antiretroviral-associated hepatotoxicity

The use of cART is associated with toxicities and since patients are on chronic treatment with cART, adverse-effects are becoming a major safety concern. In particular, ARV-associated hepatotoxicity and related diseases are becoming increasingly more prominent in HIV-infected patients [22, 23]. Hepatotoxicity is one of the foremost causes of non-AIDS related deaths and accounts for approximately 13%-18% of all-cause mortality in people infected with HIV [24]. A study done on adverse-events of ARV drugs in 33 000 patients indicated that while viral infections are a major cause of liver disease, 2.3% of total non-AIDS related deaths in this study occurred due to cART treatment [25]. Clinical findings in a retrospective study done in South America in 6000 HIV-infected patients on ART found that while co-infection with viral hepatitis may have been a contributing factor, terminal liver failure or cirrhosis is the leading cause of death in 54 out of 130 deaths in this study [26]. A cohort study in 2365 HIV-infected individuals in Switzerland, in the absence hepatitis infection, found that 16% had chronically elevated Alanine transaminase (ALAT). Prolonged ART exposure was found to be one of the risk factors associated with elevated ALAT [27].

The liver is a vital organ which is responsible for the biotransformation of xenobiotics [28]. The incidence of hepatotoxicity has been noted with the use of all current ARV drug regimens [29]. Clinical presentations of hepatotoxicity range from asymptomatic elevations of ALAT or Aspartate aminotransferase (ASAT) levels to hepatic failure in HIV-infected patients [30]. Severe disintegration of liver functioning is associated with jaundice, coagulopathy, markedly elevated ALAT levels and even death [24]. Milder cases of ARV-associated hepatotoxicity are usually reversible and are characterized by abnormal serum aminotransferase levels with or without clinical symptoms of hepatotoxicity [31]. There are

many factors which contribute to the hepatic effects of a particular drug, such as age, stage of HIV disease, comorbidities, concurrent use of alcohol as well as drug-drug interactions, hepatic metabolism and intrinsic hepatic effects [29, 32]. Mechanisms of liver injury in HIV patients caused by ARVs include mitochondrial injury, lipotoxicity, accumulation of toxic metabolites and oxidative stress [33].

In 2010 the WHO recommended the discontinuation of d4T in therapy, despite its superior virological efficacy, due to significant long term toxicity [34]. Fortuin-de Smidt M et al later reported that d4T had a 30.8 times more likelihood of experiencing treatment-limiting toxicity relative to patients on ABC [35]. The long term adverse effects associated with d4T use include lactic acidosis, severe hepatomegaly with steatosis and lipodystrophy. In addition, AZT has been associated with incidence of severe hepatotoxicity [36].

Common cART toxicities hamper the patient's ability to adhere to therapy, yet adherence is essential in order to curb the increasing incidence of drug resistance. Identification and awareness of these toxicities are vital in order to facilitate patient adherence and to determine when an intervention in therapy may be required [37].

1.4 Nucleoside/Nucleotide Reverse Transcriptase Inhibitors: Mechanisms of hepatotoxicity

NRTIs are known to cause cardiomyopathy, neuropathy, myopathies, hyperlactatemia, lactic acidosis, hepatic steatosis, insulin resistance, hepatomegaly and diabetic mellitus [38]. NRTI-associated hepatotoxicity was reported early in the epidemic and research continues to point to mitochondria as a target for injury [39, 40]. Mitochondria are responsible for the normal functioning of metabolic processes such as adenosine triphosphate (ATP) production, fatty acid metabolism, calcium signalling, cellular metabolism, steroid synthesis, heme production, apoptosis and autophagy in eukaryotic cells [41].

NRTIs have been shown to exercise toxic effects on mitochondria and the extent of damage was observed during chronic exposure to NRTIs. Studies have reported the presence of different NRTI pathogenic mechanisms [42-44]. Hepatotoxicity is prominent as the liver has a relatively large number of mitochondria due to its high energy demand. The "DNA pol-γ hypothesis" together with oxidative stress and acquired mitochondrial DNA (mtDNA) mutations form the pathophysiological mechanisms involved in NRTI-induced hepatotoxicity [45]. In order for pathophysiological events to occur, damage would have to reach threshold in order to have a detrimental impact on organ function [46].

1.5 Mitochondrial DNA polymerase-gamma hypothesis

Mitochondria possess their own DNA which encodes genes involved in the process of oxidative phosphorylation (OXPHOS), which produces energy in the form of ATP. Mitochondrial DNA (mtDNA) are more susceptible to damage possibly due to the absence of repair enzymes for mtDNA error excision, lack of histone protection, as well as mtDNA being in close subcellular proximity to reactive oxygen species (ROS) [46, 47]. Studies show that NRTIs inhibit the activity of mtDNA pol-γ (pol-γ) which is an enzyme responsible for the replication and repair of mtDNA [48-50]. These inhibitory effects are thought to be caused by the active metabolites of NRTIs. NRTIs are converted by intracellular deoxynucleotide kinases to their active moieties ("monophosphate (−MP), diphosphate (−DP), and triphosphate (−TP) nucleotides") via the endogenous deoxynucleotide phosphorylation processes (Figure 1). The phosphorylation of NRTIs differs with the tissue and mitotic state of the cell [43]. This inhibition of mtDNA pol-γ is cumulative and toxic manifestations increase with prolonged exposure. The mechanism appears to be the competition of active NRTI nucleotide with innate nucleotide pools at the nucleotide binding site of DNA pol-γ [42, 51]. Therefore, competitive inhibition of DNA pol-γ leads to reduced mtDNA copies and an increase in mutations within mtDNA. The depletion of mtDNA and

the increase in mutated mtDNA causes inhibition of synthesis of adequate proteins required for OXPHOS. Interference with OXPHOS causes energy loss (decreased ATP production) and an increase in electron leakage from electron transport chain (ETC) into the mitochondrial matrix, which leads to a rise in ROS levels. The presence of ROS causes further oxidative damage [47]. The impairment of OXPHOS and oxidative damage to cellular components in addition to the delay in cell cycle progression, ultimately causes hepatocyte apoptosis [52].

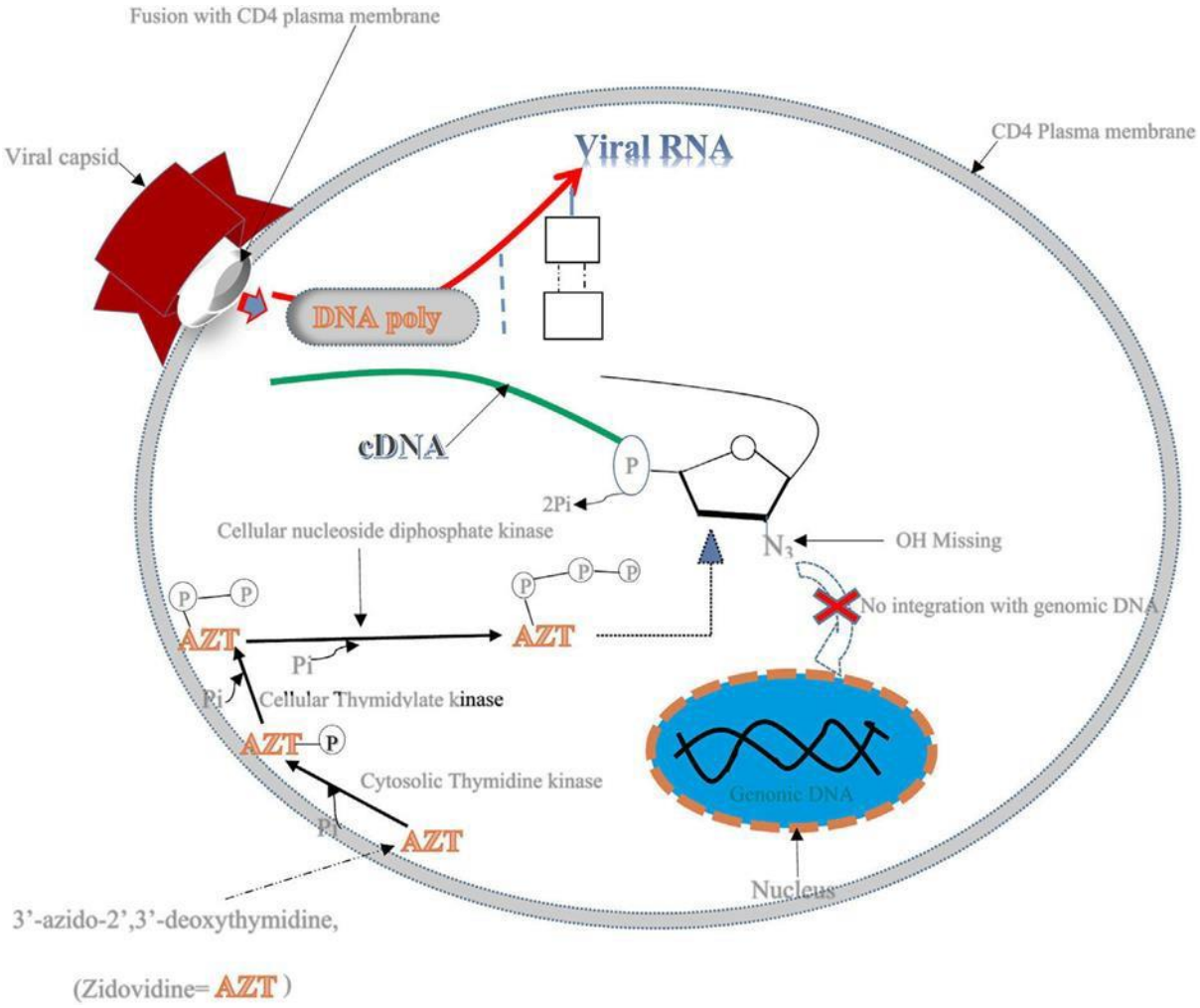


Figure 1: NRTI mechanism of action shown by AZT [53]

Moreover, NRTIs terminate nascent mtDNA synthesis, as mtDNA lack a 3-OH group on their pentose rings, which prevents the subsequent incorporation of nucleoside analogues (Figure 1). This consequently inhibits the formation of phosphodiester bond formation, and terminates mtDNA chain elongation. The continuation of mtDNA polymerization is prevented. Termination of chain elongation decreases mtDNA copies. Reduced mtDNA copies and inadequate expression of mutated mitochondrial-encoded genes involved in ETC transport further contributes to defective OXPHOS [46, 54].

The introduction of mutations into mtDNA occurs during mitochondrial polymerization. Therefore, the accumulation of faulty mtDNA results in energy depletion which leads to a pathophysiological chain of events. This seems to be supported by the presence of MDA on/near mitochondrial membrane due to cross-linking or deletion errors in transcription or mtDNA polymerization [42]. Our laboratory has reported that NRTI therapy has been associated with an increase in ROS, MDA production and carbonyl proteins (end products of protein oxidation) and decrease in antioxidant enzyme activity [38]. Furthermore, NRTIs are removed from mitochondria at a slow rate and have been found to inhibit the activity of 3'-5' exonucleases [55]. Exonucleases have proof reading activity during DNA replication and play a key role in maintaining genome stability [56]. Human pol–γ possesses 3' and 5' exonucleases which are able to remove nucleoside analogues to reduce the toxicity of a drug [57]. Studies have found that AZT-MP inhibits exonuclease functioning to a greater extent, thus pol-γ is unable to efficiently remove AZT from endogenous DNA, leading to the accumulation of high levels of AZT [46]. The accumulation of AZT in the mitochondria contributes to oxidative damage [58]. In addition, the azido group of AZT causes steric hindrance to the hydrogen bonding of Glu-200 with OH anion which leads to resistance of exonucleolytic cleavage. Moreover, faulty exonuclease activity leads to mutations in mitochondrial DNA, leading to impaired OXPHOS [59].

A study by Lund et al [43] argued that it is becoming a norm to invoke the "DNA pol-γ hypothesis" in the absence of evidence representing the complete pathogenic mechanism involved in NRTI-induced hepatotoxicity, namely mtDNA depletion, mtRNA depletion and respiratory complex dysfunction, or oxidative stress [43]. Furthermore, studies have shown that AZT significantly increases ROS production but least depleted mtDNA, while ddC and ddI significantly depleted mtDNA without significantly increasing lactate or superoxide production. AZT exposure therefore caused increased oxidative damage which correlates with previous findings of AZTs pro-oxidant effects [47, 60]. This supports claims that AZT-triphosphates have a low affinity for mtDNA pol-γ, and instead acutely induces elevation of ROS and change the functionality of respiratory complexes I and III with long term exposure [43, 60].

Studies show that AZT causes chain termination and promotes ROS production leading to mtDNA depletion and inhibition of OXPHOS [47]. Oxidative stress can disrupt mtDNA replication by oxidizing mtDNA templates. In addition, DNA- pol-γ is very susceptible to oxidative damage [44]. *In vivo* and *in vitro* studies confirm these mechanisms as NRTI treatment has shown reduced antioxidant enzyme activity as well increased MDA production [38, 45]. This accounts for AZT-induced cytotoxicity without the involvement of pol-γ. Furthermore, d4T causes disruption of the mitochondrial respirator chain which leads to lactic acidosis [50].

Therefore, while the pol-γ hypothesis is a valuable model to depict hepatotoxicity, it is not the exclusive mechanism by which NRTIs exert adverse effects. The varying degree to which NRTIs inhibit DNA pol-γ is further proof that hepatotoxicity is exhibited via additional mechanisms as well as the differences in prodrug activation in varying tissues [43, 60]. Phosphorylation of active AZT and d4T preferentially takes place in proliferating cells whereas ddC and ddI become activated in resting cells [60].

Early research done to determine the mechanism of NRTI-induced mitochondrial toxicity found NRTI-TPs to be substrates for cellular DNA polymerases. The general inhibitory effect of NRTIs on polymerases relative to HIV-Reverse Transcriptase is HIV-RT >> DNA pol c > DNA pol b > DNA pol a = DNA pol e. NRTIs have the highest affinity for DNA pol-γ [61].

The "DNA pol-γ hypothesis" suggests that various factors play a role in the ability of NRTIs to cause long term mitochondrial toxicity such as (i) tissue-specific drug penetration and metabolism, (ii) tissue polymorphism in mitochondrial DNA pol c, (iii) the target tissue stores of natural nucleotides, and (iv) the dependency of a specific tissue on mitochondrial function. The incidence of NRTI-induced hepatotoxicity varies across the range of drugs. ddC, ddI, d4T, and AZT have a higher potential of inducing mitochondrial toxicity compared to 3TC, ABC, TDF or FTC [44]. The extent to which NRTIs deplete mtDNA showed an overall rank-order of ddC > ddI > d4T >> AZT ≥ 3TC = ABC = TDF which correlates with the potential of these drugs to impede DNA pol-γ activity [60, 61]. Venoff et al showed that NRTIs can be subdivided according to their ability to inhibit liver cell proliferation with or without inhibiting pol-γ *in vitro* [62]. ddC, ddL, and d4T have a higher affinity for pol-γ and impair mtDNA replication in hepatocyte to a greater extent as well as inhibit mtDNA-encoded respiratory chain subunits interfere with cell division, enhance lipid levels and lactate production [59]. Whereas the active metabolites of TFV, 3TC, FTC, AZT and ABC show much weaker interaction with DNA pol-γ which supports earlier reports [62].

NRTIs used in combination results in increased mitochondrial toxicity. FTC 5'-triphosphate has been found to be a weak inhibitor of mammalian DNA pol-γ [59]. Toxicity studies *in vitro* support the findings that 3TC and FTC mono-exposure is essentially not toxic [62]. However, NRTIs are usually administered in combination and 3TC was found to enhance the cytotoxic effects of AZT despite the lack of mitochondrial toxicity when 3TC is administered alone [59]. Venhoff et al observed that both cytidine analogues FTC and 3TC exacerbated the

toxicity of ABC and AZT *in vitro* [62]. The study proposed that the mechanism for comparatively early toxicity of AZT could be the binding of AZT to adenylate kinase and inhibition of the ADP-ATP translocator [62]. It has been reported that AZT inhibits thymidine phosphorylation in liver mitochondria and disrupts the synthesis and availability of natural pyrimidines which compete with AZT at DNA pol-γ as well as generate ROS resulting in oxidative damage [15, 63]. A review on the mechanisms of NRTI toxicity suggests that intramitochondrial transport, compartmentalization and phosphorylation to active moieties of NRTIs by host cell also play a subcellular role in pharmacological mechanisms [47].

1.6. Pharmacogentics of Nucleoside Reverse Transcriptase Inhibitors

NRTI-induced mitochondrial toxicity is a complex phenomenon involving many factors. Genetic predisposition to hepatotoxicity is an emerging reality in pharmacogenetics. Pharmacogenetics refers to the effects of gene polymorphism on therapeutic drug outcomes. Factors such as usage of medication, gender, elevated CD4 counts and genetic factors play a role in the development of mitochondrial toxicity [64, 65]. Studies have found that ABC hypersensitivity has been strongly related to genetic allele HLA-B*57:01 [66]. NRTI uptake into the liver as well as intracellular phosphorylation influences the extent of mitochondrial toxicity [65]. Therefore, gene polymorphism on transport proteins such as p-glycoprotein (P-gp) and MRP2 and MRP4 are vital in determining intracellular concentrations of NRTIs [67]. Therefore, there is a need for additional investigations into genetic risk factors that are responsible for ARV-induced side effects such as hepatotoxicity.

1.7. Mitochondrial oxidative stress

Oxidative stress amplifies the pathophysiological and phenotypic effect of NRTI-induced mitochondrial toxicity. Oxidative stress plays a vital role in the pathophysiology of hepatotoxicity, whereby free radicals attack cell membranes, lipids and proteins resulting in

cellular and tissue injury [46, 47]. ROS are involved in the regulation of cellular functions such as gene expression for growth and death promotion, signal transduction pathways and defence against invading microorganisms [68]. Superoxide (O^{-2}), hydroxyl radicals ($OH^{\cdot}$), and peroxyl radicals as well as non-radicals such as hydrogen peroxide (H_2O_2), hypochlorous acid and o-zone are produced as by-products of normal aerobic metabolism, and are known as ROS [69]. Since liver plays an integral role in metabolic homeostasis, the high metabolic activity within the liver makes it a major target for attack by ROS [70].

Oxidative stress occurs when ROS overpowers the detoxification and scavenging ability of antioxidant defences, leading to damaged cell components [71]. Under normal physiological conditions the body has the ability to maintain homeostasis by removing ROS, in order to prevent lipid peroxidation, DNA strand breakage and indiscriminate oxidization of molecules in biological membranes. High levels of ROS induce cell death via necrotic and/or apoptotic mechanisms [69, 68, 72]. Parenchymal cells in the liver are often targets for oxidative stress-induced liver injury. Kupffer cells, hepatic stellate cells and endothelial cells have been noted to be more sensitive to oxidative stress. Additionally, a variety of cytokines such as TNF-alpha induced by oxidative stress can be produced in Kupffer cells, resulting in increased inflammation and apoptosis [71]. Furthermore, lipid peroxidation caused by oxidative stress, prompts the proliferation and collagen synthesis of hepatic stellate cells [23].

1.8 Antioxidants

Antioxidants are substances that are capable of delaying or inhibiting oxidation in low concentrations. They decrease the concentration of oxidants, neutralize metal ions to reduce the formation of ROS, convert peroxides into less reactive products, avoid initiation of chain reactions and end the formation of propagation of free radicals [68, 69]. The effects of ROS can be neutralized by enzymatic and non-enzymatic antioxidant mechanisms. These include low molecular weight compounds (such as ascorbic acid (vitamin C), alpha-tocopherol

(vitamin E) and glutathione (GSH)) and enzymes with antioxidant activity (superoxide dismutase, glutathione peroxidase and catalase) [32].

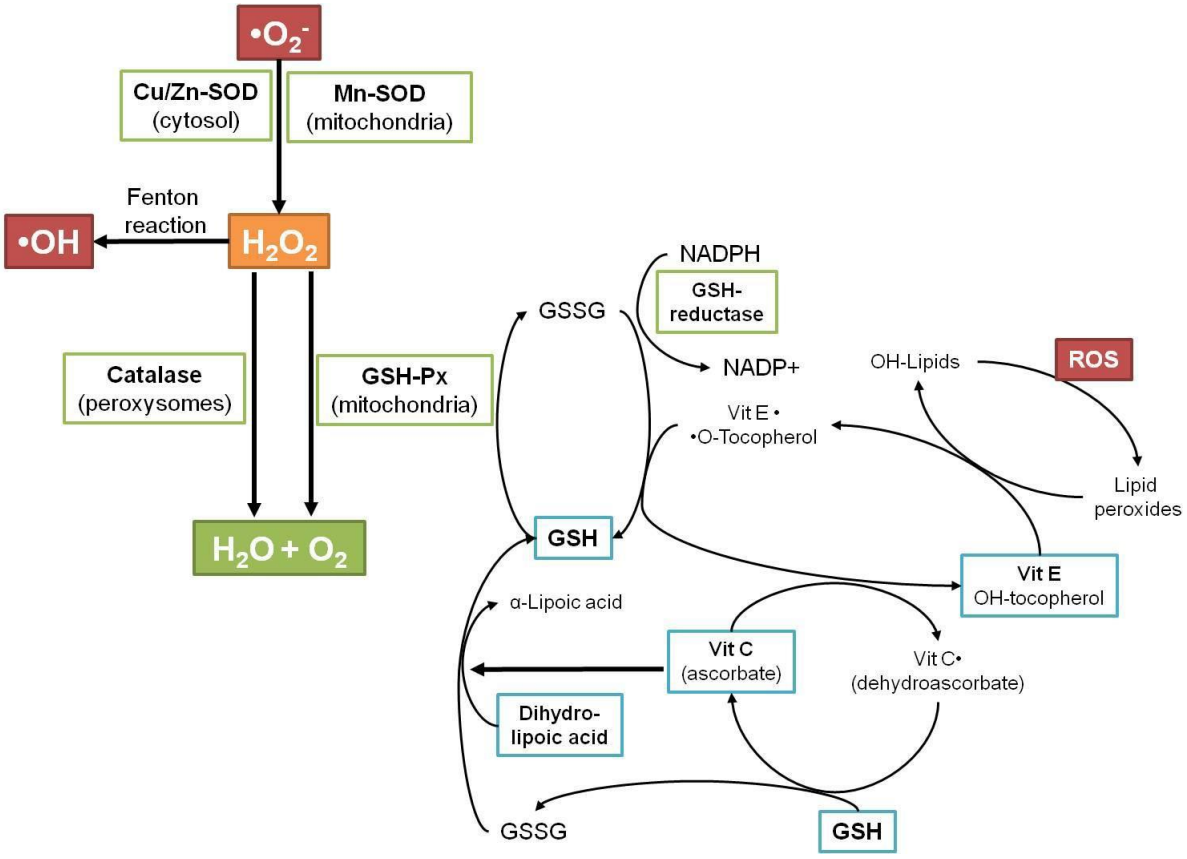


Figure 2: Antioxidant defence system [73].

Cytochrome C and carrier co-enzyme Q as well as four inner membrane associated complexes (complex I-IV) are responsible for electron flow across the mitochondrial ETC. The ETC produces ROS namely, superoxide, hydrogen peroxide and hydroxyl radical. Superoxide is continuously produced by ETC at ETC complexes I (NADH dehydrogenase) and III (umbisemiquinone). Superoxide is transformed by mitochondrial enzyme manganese superoxide dismutase (MnSOD) to hydrogen peroxide [74]. Consequently, hydrogen peroxide is converted by glutathione peroxidase (GPx) or catalase (CAT) to water molecule, resulting in the detoxification of the ROS. GPx converts H_2O_2 and other lipid peroxides to water and glutathione disulfate (GSSG) in the presence of GSH [68, 75]. GSSG is further catalysed by glutathione reductase (GST) in the presence of nicotinamide adenine

dinucleotide phosphate (NADPH) back to GSH (Figure 2). GSH is therefore an important co-factor of several detoxifying enzymes. GSH is further responsible for directly scavenging hydroxyl radicals and single oxygen to produce thiyl radicals via glutathione peroxidase; regeneration of antioxidants vitamin E and C to active forms and involvement of amino acid transport through plasma membrane. Increased generation of H_2O_2 causes the production of $OH^{\cdot}$ via Fenton reaction in the presence of ferrous (FE^{2+}) and via Haber-Weiss reaction [69, 72]. If rapid detoxification of ROS does not take place, the ROS oxidize mitochondrial components causing protein cross linkage, DNA fragmentation, phospholipid peroxidation and activation of stress and inflammatory pathways [48].

NRTIs have been reported to induce oxidative stress [48]. Inhibition of DNA polymerase-γ by NRTIs results in the production of ROS and altered expression of protein components of the respiratory chain. It has been reported that NRTIs diminish OXPHOS due to inhibition of ETC complex proteins, particularly NADH-CoQ reductase and Cytochrome c Oxidase. Cytochrome c oxidase, the fourth ETC complex, is where the final coupling of electrons occurs, leading to the formation of water and ATP [75]. Our laboratory has previously reported that NRTIs inhibit Cytochrome c oxidase production and activity, suggesting mitochondrial toxicity [38]. Studies have established that exposure of mtDNA to ROS results in extensive DNA strand breakage and degradation of deoxyribose [48]. Lewis has previously suggested that mtDNA mutations on a large scale would possibly result in inactivation of complex I as mtDNA contributes largely to the composition of the ETC complex [46]. Deficiency of complex I would result in increased superoxide production and further increase oxidative stress. Oxidation of mtDNA by hydroxyl radical leads to the formation of oxidized base 8-hydroxydeoxyguanosine, which is present at a rate 16 times greater in hepatic mtDNA than nuclear DNA. Thus, indicating that mtDNA is more prone to oxidative damage compared to nuclear DNA [46, 68].

It has been established that mitochondria produce ATP as a primary source of energy to hepatocytes. Any process which impairs mitochondrial functioning would therefore cause hepatic injury. Increased stress on the endoplasmic reticulum (ER) is the primary mechanism that leads to mitochondrial injury. This increased ER stress is triggered by various mechanisms, with a final common pathway which results in apoptosis and increased macrophage activation and beta-oxidation of accumulated fatty acids within the liver and increased production of inflammatory cytokines [76]. In addition, NRTIs directly cause mitochondrial toxicity by increasing lipid content of cell membranes leading to ER stress resulting in mitochondrial dysfunction [64, 76]. Lipotoxicity in the liver is due the deposition of free fatty acids (FFA). Peroxidation of these FFA leads to increased ROS and ER stress which results in liver damage and fibrosis [77, 78].

1.9 Naringenin

Naringenin (4′,5,7-trihydroxyflavanone) is a natural flavanone found in grapefruit, tomatoes and related citrus species. Naringenin possesses diverse biological and pharmacological properties such as, anti-apoptotic, anti-diabetic, neuroprotective, hepatoprotective, anti-inflammatory, metal chelating, anti-microbial, anti-mutagenic, anti-cancer, free radical trapping, and antioxidant properties [79-82]. The distinct bitter taste of grapefruit juice is attributed to naringenin [83]. Moreover, naringenin has been shown to have no known toxicity [84]. Studies on the acute toxicity of naringin have found that a dose of *Carissa carandas* extract, which has a high content of naringin, at 5000 mg/kg showed no deaths or toxic symptoms in rats [85]. When naringin is administered orally, it is hydrolysed in the gut by alpha- rhamnosidase and beta-glucosidase enzymes to yield an absorbable metabolite, naringenin [75].

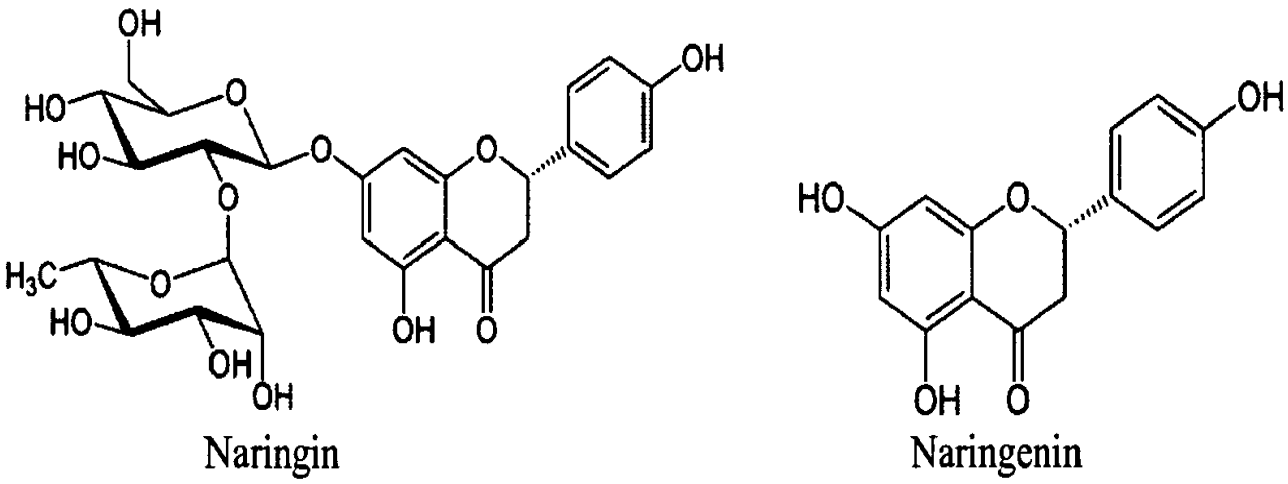


Figure 3: Chemical structures of naringin and naringenin [86].

Once naringin is hydrolysed by enteric microflora to aglyclone naringenin, it is subsequently absorbed in the intestinal barrier and metabolized by intestinal and liver cells to glucuronides and sulphoglucuronides [87, 88]. A review by Bharti et al [89] highlighted that naringin is concentrated in the liver and bile due to active transport. Research shows naringenin to be readily absorbed into cells by transcellular diffusion, due to its lipophilic nature [89]. Naringenin is also transported via an energy-dependent system mediated by multidrug resistance-associated protein 1 (MRP1) carriers and has an affinity for active efflux protein carriers P-glycoprotein (P-gp) and MRP2 [90]. Hernandez-Aquino et al [91] report that in the liver, naringenin enters hepatocytes via organic anion transporting protein-B (OATP -B) and is conjugated by UDP-glucuronosyl transferase (UGT), sulfotransferase and catechol-O-methyltransferase to naringenin-glucuronides before entering the blood circulation [91]. However, a study by Chabane et al [90] found that naringenin is not a substrate for OATP-B but rather modulates the expression of this protein carrier resulting in drug or food interactions. Interestingly, an *in vivo* study [80] using a rabbit model reported naringenin to possesses poor oral bioavailability of 4%. The hydrophobic ring structure accounts for the low solubility and minimal bioavailability [92]. In addition, it has been demonstrated that

naringenin has a low bioavailability of 5.8% after 3.5 h of administration of 135 mg naringenin to six healthy volunteers which was attributed to the inter-individual variation in gut flora [88].

Naringenin has three hydroxyl substitutions which are capable of donating hydrogen to free radicals, thus scavenging ROS. Naringenin is stabilized by resonance due to the presence of aromatic, heterocyclic rings and multiple unsaturated bonds [93]. The B ring in the flavonoid structure can stabilize the hydroxyl, peroxyl and peroxynitrite radicals to form a stable flavonoid radical (Figure 3). The relationship between 5-OH and 4-oxo plays an integral role in naringenin's ability to chelate heavy metal compounds [91]. Naringenin lacks the sugar moiety present on naringin, and is therefore more potent than naringin, as the sugar moiety causes steric hindrance to the scavenging group [83].

Naringenin is known to scavenge ROS, prevent free radical attack, thus improve oxidative stress [94, 95]. Oxidative stress amplifies the pathophysiological and phenotypic effects of NRTI-induced hepatotoxicity. Oxidative stress plays a vital role in the pathophysiology of hepatotoxicity, whereby free radicals attack cell membranes, lipids and proteins resulting in hepatocyte and tissue injury [72, 71]. Naringenin has been noted to upregulate SOD, CAT, GPx and GST enzymes [87, 96]. This is consistent with the hypothesis that naringenin increases protein content and mRNA levels of hepatic antioxidant enzymes which is essential for enzyme production [91]. Naringenin further increases GSH/GSSG ratio [96]. GSH is a non- enzymatic antioxidant which detoxifies ROS, and is involved in thiol disulphide exchange and storage and transfer of cysteine. GSH is therefore an important co-factor of several detoxifying enzymes. In addition, GSH is responsible for scavenging hydroxyl radicals and single oxygen directly producing thiyl radicals via GPx; regeneration of antioxidants vitamin E and C to active forms and involvement of amino acid transport through plasma membrane [72].

Another proposed mechanism for naringenin's antioxidant effects is its ability to reduce cell membrane fluidity due to its lipophilic interaction with the polar heads of phospholipids. This lipophilic interaction stabilizes the cell membrane and decreases the interaction of free radicals and lipids, thus ameliorating lipid peroxidation [97] and protecting the cell membrane [75, 71]. Multiple studies have shown that naringenin is capable of reducing lipid peroxidation in the liver [94, 96, 98]. Therefore, the beneficial effects of naringenin against lipid peroxidation is an important consideration, as NNRTIs, NRTIs among other ARVs initiate lipid peroxidation, therefore triggering liver injury.

Previous studies have shown that naringenin can protect against hepatotoxicity induced by arsenic by decreasing oxidative stress and also prevents ethanol-induced hepatotoxicity in a rat model [83 95]. Our laboratory investigated the antioxidant and free radical scavenging effects of naringin in mitochondrial toxicity caused by NRTIs *in vivo* [38]. NRTIs were noted to increase the production of mitochondrial ROS leading to the damage of lipids, amino acids and DNA components, which resulted in the formation of protein carbonyls, 4-hydroxynonenal, malondialdehyde (MDA) and advanced glycation end products, among others. Treatment with naringin however, significantly reduced MDA concentrations, and prevented the depletion of antioxidant enzyme activity and attenuated lipid peroxidation [38], similar to reports in other studies [45, 80, 71]. In addition, naringenin was found to protect against NRTI-induced apoptosis of hepatocytes. The study hypothesized that naringin improves the expression and/or the activity of Nuclear factor-erythroid 2 related factor (Nrf2). Nrf2 is regulates antioxidant enzyme gene expression and plays a role in regulating the level of cellular oxidative stress. Consequently, increased expression of Nrf2 levels would reduce oxidative stress [72, 71].

1.9.1 Nuclear factor-erythroid 2 related factor hypothesis

Naringenin has been reported to reduce pro-inflammatory mediators such as nitric oxide synthase (iNOS), cyclooxygenase-2 (COX-2), and Tumor necrosis factor (TNF-α) via upregulating the anti-oxidative defence proteins heme oxygenase (HO-1) and Nrf2 [87, 99]. In addition, naringenin showed anti-inflammatory properties as it maintained levels of nuclear factor kappa-light-chain-enhancer of activated B cells (NF-kB), Interleukin-1 (IL-1) and IL-10, which are key proteins involved in inflammation [75]. It has been reported that naringenin is capable of upregulating Nrf2 mRNA expression, thus resulting in an increase in Nrf2 proteins, which leads to transcriptional activation of detoxification and antioxidant proteins [38, 91]. Inactive Nrf2 in the cytoplasm interacts with Kelch-like ECH associating protein 1 (Keap1). Once released by Keap1, Nrf2 becomes active either by MAPK phosphorylation or by conformational change in Keap1 due to the presence of ROS. Free Nrf2 translocates to the nucleus to form a transcriptional complex with musculoaponeurotic fibrosarcoma (Maf) family proteins. This dimer binds to the promoter region of antioxidant response element (ARE) sequence resulting in coordinated transcriptional activation of antioxidant enzymes and detoxifying proteins, such as NQO1, GST and HO-1, GPx, CAT, SOD. Therefore, activation of Nrf2 may be a novel strategy to attenuate hepatotoxicity as Nrf2 is vital for the activation of detoxification genes and preventing oxidative stress [91, 94]. Esmaeili et al [94] suggested that naringenin treatment enhanced nuclear expression of Nrf2 leading to increased activity of antioxidant enzymes in rats treated with CCl_4 [94]. However, a review by Hernandez-Aquino et al [75] argued that increased Nrf2 protein levels may not necessarily translate to increased Nrf2 activity as Nrf2 must disassociate from Keap1 and translocate to the nucleus in order to activate antioxidant defence systems. Therefore, naringenin may enhance translocation of Nrf2 to the nucleus from the cytoplasm [75]. A study using cardiomyocytes corroborated that naringenin increased phosphorylation and

translocation of Nrf2 to the nucleus and increased the mRNA expression of c-glutamyl cysteine as well as glutamyl cysteinyl ligase (GCL) resulting in increased expression of antioxidant GSH [99]. Moreover, naringenin was reported to increase mRNA and protein levels of antioxidant gene proteins [79].

Naringin supplementation was found to protect against liver injury in a high-fat-diet-fed rat model, possibly mediated by activation of AMPK, which restored antioxidant enzymes and prevented inflammation. Naringin was found to reduce the release of TNF and improve liver damage. It has been reported that naringin scavenges ROS and modulates expression of c-glutamylcysteine synthetase, the rate limiting step in the synthesis pathway of GSH [100]. GSH acts as a nucleophile which reacts with electrophilic species hence, and increase in GSH concentrations would translate to protection against oxidative damage [80, 100].

1.9.2 Naringenin and hepatocyte apoptosis

Apoptosis is known as programmed cell death. It is an endogenous defence mechanism used to remove cells that are damaged, left unchecked however, apoptosis can lead to pathological conditions [70]. Apoptosis is a highly complex energy-dependent process which involves the activation of caspases which ultimately result in a cascade of events which lead to cell death [101]. An apoptotic cell generally appears smaller in size with a dense cytoplasm and tightly packed organelles. Most cells express caspases in an inactive proenzyme form. Caspase activation leads to activation of other procaspases which amplifies the apoptotic signalling pathway, thus causing rapid cell death. Caspase-2, -8, -9, and -10 are known as initiators while caspase-3, -6, -7 are known as executioners. Execution caspases are responsible for the morphological changes of apoptotic cells [102]. Caspase-3 is known as the most important caspase as it is activated by initiator caspase, which activates CAD resulting in degradation of chromosomal DNA. Hepatocytes may undergo apoptosis by intrinsic or extrinsic pathways [91].

The extrinsic pathway involves the activation of the death domain which transmits the death signal from the death receptors on the cell surface to the intracellular signalling pathways. Upon binding of death ligands (TNF- α, TNF-related apoptosis-inducing ligand (TRAIL) and FasL) to receptors, conformational change occurs which recruits cytoplasmic adaptor proteins (Fas-associated protein with death domain (FADD)) and TRADD. FADD then associates with procaspase-8 which consequently forms a death-inducing signalling complex (DISC) thus leading to the activation of execution caspase cascade, ultimately leading to apoptosis [70, 102, 103].

The intrinsic pathway is activated by stimuli, such as ROS. These stimuli cause a change in mitochondria intermembrane space which releases pro-apoptotic proteins via mitochondrial permeability transition pores, into the cytoplasm. Pro-apoptotic protein cytochrome c bind to and activate Apaf-1 and procaspase-9 forming an apoptosome which activates the caspase cascade. The intrinsic pathway is regulated by Bcl-2 family proteins, namely anti-apoptotic proteins Bcl-2, Bcl-x, Bcl-XL and pro-apoptotic proteins Bcl-10, Bax, Bak and Bad. Anti-apoptotic proteins abort apoptosis by altering mitochondrial membrane permeability thus preventing the release of electron transport protein cytochrome c from the mitochondria [91]. Pro-apoptotic proteins translocate to mitochondria and aid the release of cytochrome c [70]. It has further been reported that Bcl-2 and Bcl-XL inhibit apoptosis by controlling activation of caspase proteases [102].

Increased levels of ROS has been associated with oxidative damage to mitochondrial DNA, thus inducing hepatocyte apoptosis [70]. Oxidative stress causes altered expression and signal transduction of pro-apoptotic and anti-apoptotic proteins [104]. Treatment with naringin reversed ROS-mediated apoptosis by caspase-3 activation in human polymorphonucleur neutrophils. Naringin was further found to inhibit apoptotic markers Bax and Bad in a rodent

model [89]. Moreover, naringenin was found to increase the expression of anti-apoptotic protein Bcl-2, thus protecting against apoptosis [104].

Treatment with naringenin in an *in vitro* model was found to reduce apoptotic markers caspase-3, -8 and-9 [87, 99]. Another *in vivo* study by our laboratory found that naringin improved hepatocyte apoptosis and oxidative stress associated with NTRI-induced metabolic complications [45]. The study found apoptotic features upon ultrastructural examination of hepatocytes following AZT and d4T alone treatment. Naringin minimized mitochondrial damage and further mitigate the AZT and d4T-induced hepatocyte apoptosis by significantly decreasing the expression of pro-apoptotic protein Bax and significantly elevating the expression of anti-apoptotic protein Bcl-2 [45]. Kapoor et al [105] reported that ROS activates Bax resulting in the translocation of Bax to the outer mitochondrial membrane. This causes a decrease in mitochondrial membrane potential, resulting in mitochondrial membrane leakage, which triggers caspases and other pro-apoptotic agents, ultimately increasing the rate of apoptosis. Anti-apoptotic Bcl-2 conversely prevents apoptosis by binding to and inhibiting agents that promote apoptosis [105].

Naringin could be a useful supplement in protecting NRTI-induced hepatocyte apoptosis. This is in agreement with a recent study which suggested that naringenin significantly reduced the activity of caspase-3 activity induced by isoniazid (INH) and rifampicin (RIF) treatment in hepatocytes [106]. Naringenin was found to upregulate anti-apoptotic Bcl-2 expression and downregulate pro-apoptotic Bax expression, thus markedly reducing hepatocyte apoptosis induced by INH and RIF. In addition, our laboratory reported that naringin supplementation prevented PI-induced apoptosis and reduced metabolic complication associated with PIs. Naringin was found to markedly reduce the activity of caspase-9 in rodents treated with SQV and ATV [107].

Naringenin can modulate hepatic expression of transporting protein OATP which may be involved in the metabolism of many drugs, thus drug-drug interaction is an important consideration which requires further investigation. Furthermore, HIV infection alone is associated with production of free radicals which cause oxidative damage and induce apoptosis by CD4 T cells [108]. Therefore, naringenin has demonstrated antioxidant, and anti-apoptotic properties that may be useful in ameliorating NRTI-induced hepatocyte injury.

1.10 Hypothesis

Naringenin attenuates NRTI-induced oxidative stress and apoptosis in Chang cells *in vitro*.

1.11 Aims and objectives

1. To investigate whether naringenin could reverse NRTI-associated oxidative stress in hepatocytes *in vitro*.

2. To investigate whether naringenin could reverse NRTI-associated apoptosis in hepatocytes *in vitro*.

CHAPTER TWO

Materials and methods

2.1 Chemicals and reagents

Chang Liver cells were purchased from ATCC (Virginia, USA). NRTIs (d4T and AZT) were purchased from local pharmacy. Naringenin was purchased from Sigma Chemical (St Louis, MO). Superoxide Dismutase (SOD) assay, Catalase (CAT) assay, Glutathione peroxidase (GPx) assay, Cell viability assay, Caspase-3 and -9 assay was purchased from Whitehead Scientific (Johannesburg, South Africa).

2.2 Experiment

2.2.1 Cell Culture

Chang liver cells were cultured in Eagle's minimal essential medium (EMEM) containing 5.5 M glucose supplemented with 10% Foetal Bovine Serum (FBS), streptomycin 100 µg/ml and penicillin 100 units/ml maintained at 37°C in humidified air with 5% CO_2. Cells were plated in 75 cm^3 culture flasks and subjected to treatment once the cells reached 80% confluence. Fresh cell culture medium was replenished every 24 hours after plating.

2.2.2 Trypsinisation and cell counting

Trypsinisation process is done to remove adherent cells from the culture surface after the cells became 80% confluent, as well as to subculture and seed the cells for various biochemical assays. Tripsinisation involves washing the cells twice with 0.1M PBS warmed to 37°C and incubating the cells with 1ml of trypsin-EDTA for 2 minutes. The cells were thereafter observed for detachment using an inverted light microscope (Olympus IXSI; 20 x). Once detachment was observed the trypsinisation reaction was terminated by the addition of ~ 5ml cell culture medium (CCM) containing FBS into the flask. The cells were aspirated and transferred to a 15 ml conical tube and centrifuges at 200 g for 5 minutes. The supernatant

was carefully discarded without disturbing the cell pellet. The cells were re-suspended using CCM and the homogenized suspension was calculated using a haemocytometer. Trypan blue (0.4%) was applied in a dye exclusion procedure in order to conduct a cell count. The concept of dye exclusion is built on the principle that sustainable cells possess intact cell membranes that exclude dye and remain unstained while damaged or dead cell membranes permit the entry of dye and stain blue.

2.2.3 NRTI and naringenin preparation

d4T and AZT were prepared in dimethyl sulfoxide (DMSO) at stock solution of 3 mg/ml (13.4 mM), and 1.0 mg/l (3.7 mM) respectively. Naringenin was dissolved in DMSO to a final concentration of 5 mM.

2.2.4 Cell exposure

2.2.4.1 Effects of NRTIs on cells

To measure the effects of NRTIs on Chang liver cells, the cells were plated in 75 cm^3 culture flasks (5.0×10^5 cells/flask) and grown to 80% confluence. Thereafter the cells were treated with d4T (4.0 μM), and AZT (7.1 μM), which correspond to steady state peak plasma concentration of patients receiving NRTI therapy [109], for 24 hours.

2.2.4.2 Effects of naringenin on NRTI-exposed cells

To measure the role of naringenin relative to NRTI-exposed cells, the cells exposed to d4T (4.0 μM), and AZT (7.1 μM) were consequently treated with or without naringenin (0.0, 10.0, 20.0, 30.0, 40.0 and 50.0 μM) in culture medium for 24 hours.

2.3 Biochemical assays

For SOD assay, GPx assay, CAT assay, and Caspase-3 and -9 Assay the cells were grown to 90% confluence in 75 cm^3 flasks and treated with test compounds. For 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay the cells were plated in

a 96- well microtiter plate, treated with test compounds and left to attach overnight. The cells were harvested and treated according to manufactures instructions for each assay for biochemical analysis.

2.3.1 Cell viability assay

MTT assay is a useful assay to measure cell proliferation and cell viability. This colometric assay uses the reduction of tetrazolium salt MTT to MTT formazan to determine cell metabolic activity. Viable cells contain NAD(P)H-dependent oxidoreductase enzymes which reduce MTT reagent to formazan, thus producing a characteristic purple colour. Thereafter, formazan crystals are dissolved by a solubilizing agent and absorbance is measured by a microtiter plate reader at 500-600nm. A darker solution is an indication that cells are viable and metabolically active. Cells (10 000) were seeded into a 96-well plates, treated with NRTIs with or without naringenin and incubated for 24 h at 37 °C. Media was removed and cells were rinsed with PBS. Stock MTT solution of 5.0 mg/ml (20 µl) was added to each well and incubated for 4h until purple formazan crystals appeared under the microscope. MTT solution was discarded and solubilizing solution (DMSO 100 µl) was added to each well. This was incubated at 37 °C for 1 h until the purple crystals completely dissolved. Absorbance was read at 540 nm with a EZ 400 microplate reader (Biochrom Ltd, Cambridge, UK) and calculations were done to determine cell viability.

2.3.2 Superoxide dismutase assay

SOD is an important antioxidant enzyme responsible for catalysing the reduction of superoxide anion into hydrogen peroxide or molecular oxygen. The SOD assay used in this study uses WST-1 method which produces a water-soluble formazan dye when the superoxide anion is reduced. The rate at which superoxide anion is reduced is directly related to the xanthine oxidase (XO) activity. This is inhibited by SOD. Therefore, the inhibition of

SOD activity can be determined via colorimetric method. Using a commercial kit, following treatment, the Chang liver cells were trypsinized and centrifuged for 10 min at 1000 r/min. The supernatant was discarded and cells were resuspended with 1 ml of cold 0.1M Phosphate buffered saline (PBS), centrifuged and supernatant was discarded. Cold PBS was used to resuspend cells and cells were sonicated 3 times for 30 secs respectively with hand-operated in an ice water bath to break the cells. The homogenized solution (20 µl) which contained total SOD activity from cytosolic and mitochondria, was added to each well in a 96-well microplate. Substrate applied solution and enzyme working solution was prepared according to assay protocol. Substrate applied solution (200 µl) was added to the well followed by 20 µl of enzyme working solution and incubated at 37 °C for 20min. Absorbance was read at 450 nm with a EZ 400 microplate reader (Biochrom Ltd, Cambridge, UK) and the % of SOD activity was calculated according to protocol. Sample concentrations were extrapolated using the standard curve and values were expressed in units of enzyme normalized to cellular protein in milligram (mg).

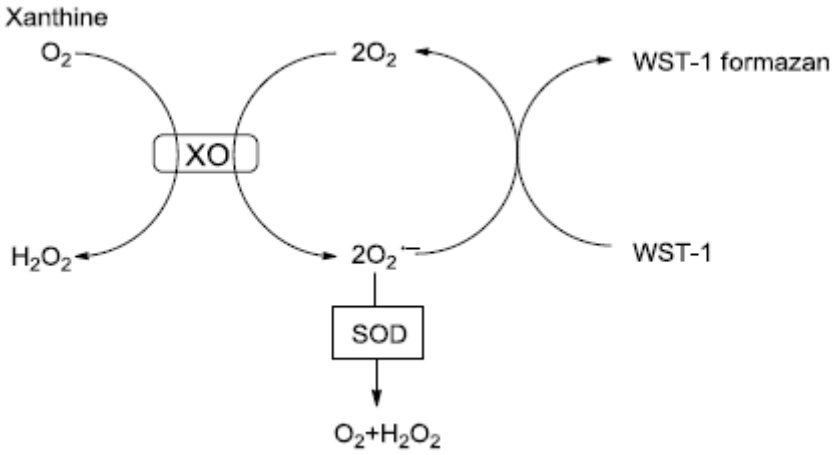


Figure 4: The principle of determining SOD activity by SOD assay (Elabscience protocol).http://www.elabscience.com/p-superoxide_dismutase_(t_sod)_colorimetric_assay_kit_(wst_1_method)-40320.html

2.3.3 Catalase assay

Catalase is a vital antioxidant enzyme which is responsible for catalysing the reduction of H_2O_2 to water and oxygen. The assay is based on the principle that the decomposition of H_2O_2 by catalase can be quickly stopped by ammonium molybdate. catalase initially reacts with hydrogen peroxide to produce water and oxygen. The unconverted H_2O_2 reacts with ammonium molybdate to produce a yellow complex which that can be measured at 405 nm (Colorimetric method). Using a commercial kit, following treatment, the Chang liver cells were trypsinized and centrifuged for 10 min at 1000 r/min. The supernatant was discarded and cells were resuspended with 1 ml of cold 0.1M PBS, centrifuged and supernatant was discarded. Cold PBS was used to resuspend cells and cells were sonicated 3 times for 30 secs respectively with hand-operated in an ice water bath to break the cells. The homogenized solution was collected (0.1 ml) and added to 0.5 cm cuvette. Assay buffer solution (1 ml) and 0.1 ml of substrate solution was added, mixed fully and incubated at 37°C for 1 min. Thereafter 1 ml of chromogenic agent and 1 ml of clarificant was added into the sample, mixed and incubated at 25°C for 10 min to inhibit the activity of catalase in samples. Absorbance first set to zero using ddH2O and measured at 405 nm with a GENESYS 20 spectrophotometer (Thermo Fisher Scientific, Massachusetts, USA). Calculations were performed to measure catalase activity.

2.3.4 Glutathione peroxidase assay

GPx is an enzyme which catalyses the reduction of hydrogen peroxide using GSH to produce water and oxidized GSH. Thus, protecting the cell membrane structure and maintaining membrane function integrity. The activity of GPx can be expressed via the rate of enzymatic reaction. H_2O_2 and reduced GSH can react in the absence of GPx. Therefore, the amount of GSH reduced via non-enzyme reaction can be subtracted. Thus the activity of GSH can be measured the consumption of reduced GSH. In this assay, GSH reacts with dinitrobenzoic

acid to produce 5-thio-dinitrobenzioc acid anion which produces a stable yellow colour. Absorbance is measured using a spectrophotometer at 412 nm. The cells were seeded (1 x 10^6), centrifuged at 1000 r/min, and 0.4 ml PBS was added to cell sediment before being sonicated in an ice water bath 3 times for 30 secs respectively. Thereafter protein concentration was detected. Optimal dilution factor of samples was determined. To the non-enzyme tube 0.2 ml of GSH was added and pre-heated for 5 min. Thereafter, 0.1 ml of reagent 1 application solution was added. This was reacted for precisely 5 min at 37 °C. Reagent 2 application solution (2 ml) was added, followed by 0.2ml of sample. To the enzyme tube, 0.2 ml of GSH and sample was added and pre-heated for 5min. 0.1 ml of reagent application solution was added and reacted at for precisely 5 min at 37 °C. Reagent 2 application solution (2 ml) was then added. Both non-enzyme and enzyme tubes were mixed fully and centrifuged for 10 min. Thereafter, 1 ml of supernatant was removed for chromogenic reaction. To the blank tube 1 ml of GSH standard application solution, 1 ml of reagent 3 application solution, 0.25 ml of reagent 4 and 0.05 ml of reagent 5 application solution was added. To the standard tube 1 ml of GSH standard solution, 1 ml of reagent 3 application solution, 0.25 ml of reagent 4 and 0.05 ml of reagent 5 application solution was added. To the non-enzyme tube 1 ml of supernatant, 1 ml of reagent 3 application solution, 0.25 ml of reagent 4 and 0.05 ml of reagent 5 application solution was added. To the enzyme 1 ml of supernatant, 1 ml of reagent 3 application solution, 0.25 ml of reagent 4 and 0.05 ml of reagent 5 application solution was added. All tubes were mixed fully and left to stand for 15 min at room temperatures. OD values were first set to zero with double-distilled water and measured at 412 nm with a GENESYS 20 spectrophotometer (Thermo Fisher Scientific, Massachusetts, USA) with 1 cm diameter cuvette. Calculations were performed to measure glutathione peroxidase activity

2.3.5 Caspase-9 enzyme-linked immunosorbent assay (ELISA) assay

Activation of caspase enzyme is responsible for initiation of apoptosis in mammalian cells. Cellular caspase -9 activity was measured using an ELISA assay kit which detects antibodies for specific human Caspase 9. Standards or samples are added to the micro ELISA plate together with specific antibodies. A biotinylated detection antibody specific for Human CASP9 and Avidin-Horseradish Peroxidase (HRP) are then added successively and incubated. Free components are washed away and substrate solution is added to each well. The wells which contain Human CASP9, biotinylated antibody and Avidin-HRP conjugate will appear blue in colour. Stop solution is added to terminate the reaction which results in the formation of a yellow solution. The absorbance read at 450 nm is proportional to the concentration of Human CASP9. Cell samples were washed with PBS, trysinized and centrifuges for 5 min at 1000 r/min. Supernatant was discarded and washed 3 times with PBS. Cells were resuspended in PBS and the freeze-thaw process was done to lyse the cells. Cells were centrifuged for 10 min at 1500 r/min at 4 °C and supernatant was collected. Reagents were prepared according to assay protocol. Sample or standard (100 µl) was added to each well and incubated for 90 min at 37 °C. Liquid was removed and 100 µl of Biotinylated Detection Ab working solution was added and incubated for 1 h at 37 °C. This was aspirated and washed 3 times before the addition of 100 µl of HRP Conjugate. This was incubated for 1 h at 37 °C. Thereafter the sample was aspirated and washed 5 times. Substrate reagent (90 µl) was added and incubated for 15 min 37 °C. Stop solution (50 µl) was added and OD was determined immediately at 450 nm using an EZ 400 microplate reader (Biochrom Ltd, Cambridge, UK). Calculations were performed to measure caspase activity by comparing OD of the samples to the standard curve.

2.3.6 Caspase-3 ELISA assay

Activation of caspase enzyme is responsible for initiation of apoptosis in mammalian cells. Cellular caspase -3 activity was measured using an ELISA assay kit which detects antibodies for specific human Caspase 3. Standards or samples are added to the micro ELISA plate together with specific antibodies. A biotinylated detection antibody specific for Human CASP3 and Avidin-HRP are then added successively and incubated. Free components are washed away and substrate solution is added to each well. The wells which contain Human CASP3, biotinylated antibody and Avidin-HRP conjugate will appear blue in colour. Stop solution is added to terminate the reaction which results in the formation of a yellow solution. The absorbance read at 450 nm is proportional to the concentration of Human CASP3. Cellular caspase -3 activity was measured using an ELISA assay kit which detects antibodies for specific human Caspase 3. Cell samples was washed with PBS, trysinized and centrifuges for 5 min at 1000 r/min. Supernatant was discarded and washed 3 times with PBS. Cells were resuspended in PBS and the freeze-thaw process was done to lyse the cells. Cells were centrifuged for 10 min at 1500 r/min at 4 °C and supernatant was collected. Reagents were prepared according to assay protocol. Sample or standard (100 µl) was added to each well and incubated for 90 min at 37 °C. Liquid was removed and 100 µl of Biotinylated Detection Ab working solution was added and incubated for 1 h at 37 °C. This was aspirated and washed 3 times before the addition of 100 µl of HRP Conjugate. This was incubated for 1 h at 37 °C. Thereafter the sample was aspirated and washed 5 times. Substrate reagent (90 µl) was added and incubated for 15 min 37 °C. Stop solution (50 µl) was added and OD was determined immediately at 450 nm using an EZ 400 microplate reader (Biochrom Ltd, Cambridge, UK). Calculations were performed to measure caspase activity by comparing OD of the samples to the standard curve.

2.4 Statistics

Experiments were done in triplicate and all data presented as mean ± SD. Statistical differences were determined by One Way Analysis of Variance (ANOVA) followed by multiple testing where applicable using Graph Pad Prism® Software version 8.0. A p-value of $p < 0.05$ was considered statistically significant.

CHAPTER THREE

Results

3.1 Antioxidants assay

3.1.1 Superoxide dismutase (SOD) assay

Total SOD activity in AZT and d4T exposed cells was significantly ($p < 0.05$) decreased compared to untreated control (Figure 5, 6). Treatment with concentrations of 40, 50 µM naringenin significantly ($p < 0.05$) increased SOD activity in AZT exposed cells compared to cells treated with AZT only. Treatment with increasing concentrations of 10 - 40 µM naringenin significantly ($p < 0.05$) increased SOD activity in d4T exposed cells compared to d4T only.

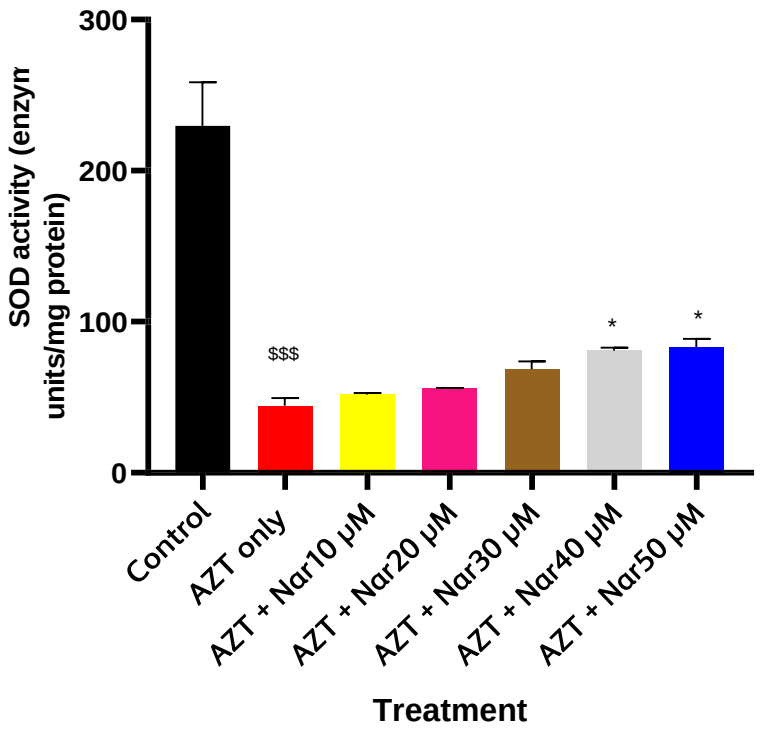


Figure 5: SOD activity in cells treated with AZT and/or different concentrations of naringenin. ($^{\$}p < 0.05$ when compared to control, $*p < 0.05$ when compared to AZT only)

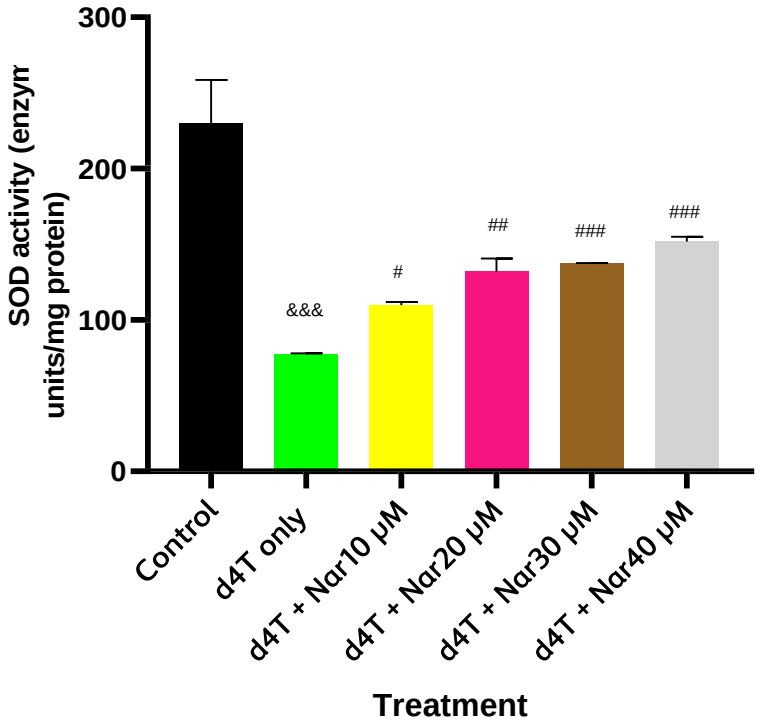


Figure 6: SOD activity in cells treated with d4T and/or different concentrations of naringenin. ($^{\&}$ $p < 0.05$ when compared to control, $^{\#}$ $p < 0.05$ when compared to d4T only)

3.1.2 Catalase activity

The catalase activity in AZT and d4T exposed cells was significantly ($p < 0.05$) decreased compared to the untreated control (Figure 7, 8). Treatment with increasing concentrations of 10 - 50 µM naringenin significantly ($p < 0.05$) increased catalase activity in AZT exposed cells compared to cells treated with AZT only. Treatment with increasing 10 - 40 µM concentrations of naringenin significantly ($p < 0.05$) increased catalase activity compared to cells treated with d4T only.

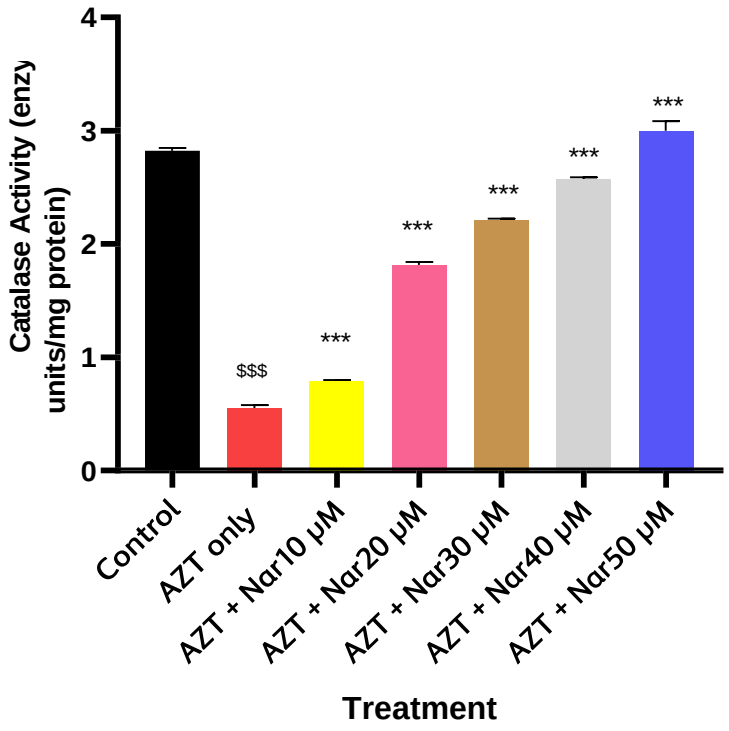


Figure 7: Catalase activity in cells treated with AZT and/or different concentrations of naringenin. ($^{\$}p < 0.05$ when compared to control, $*p < 0.05$ when compared to AZT only)

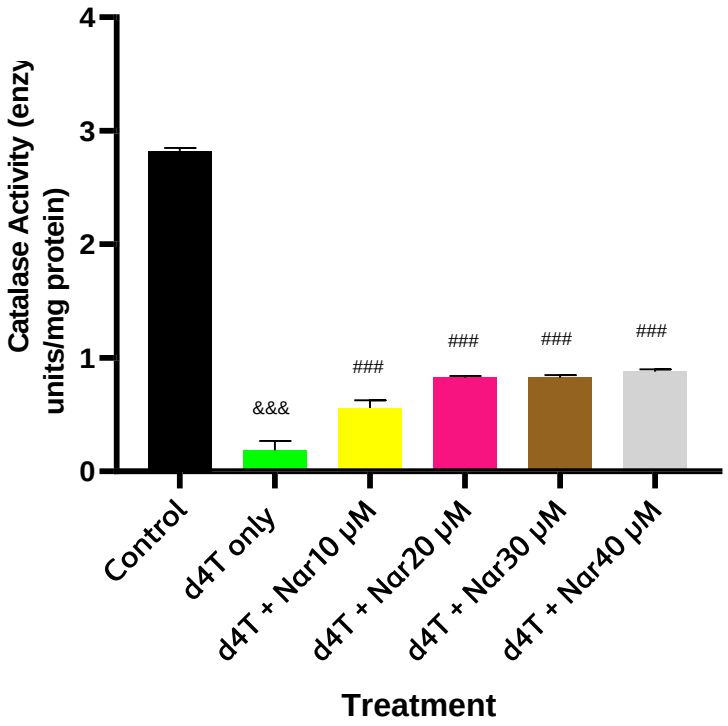


Figure 8: Catalase activity in cells treated with d4T and/or different concentrations of naringenin. ($^{\&}$ $p < 0.05$ when compared to control, $^{\#}$ $p < 0.05$ when compared to d4T only)

3.1.3 Glutathione-peroxidase (GPx) activity

The GPx activity in AZT and d4T exposed cells was significantly ($p < 0.05$) decreased compared to the untreated control (Fig 9, 10). Treatment with increasing concentration of 50 µM naringenin exhibited a significant ($p < 0.05$) increase in GPx activity in AZT exposed cells compared to cells treated with AZT only. In addition, treatment with increasing concentrations of 10 - 40 µM naringenin significantly ($p < 0.05$) increased GPx activity compared to cells treated with d4T only.

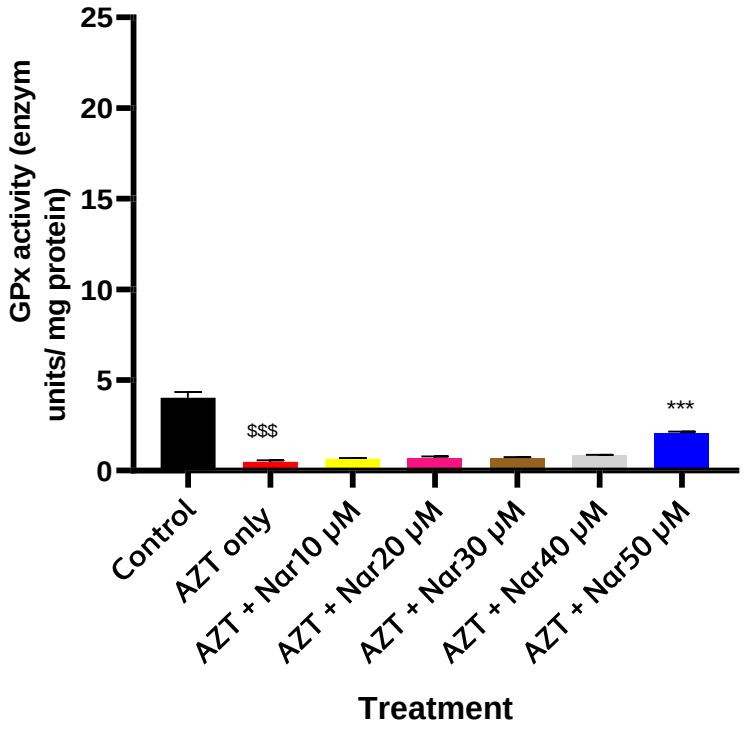


Figure 9: GPx activity in cells treated with AZT and/or different concentrations of naringenin. ($^{\$}p < 0.05$ when compared to control, $*p < 0.05$ when compared to AZT only)

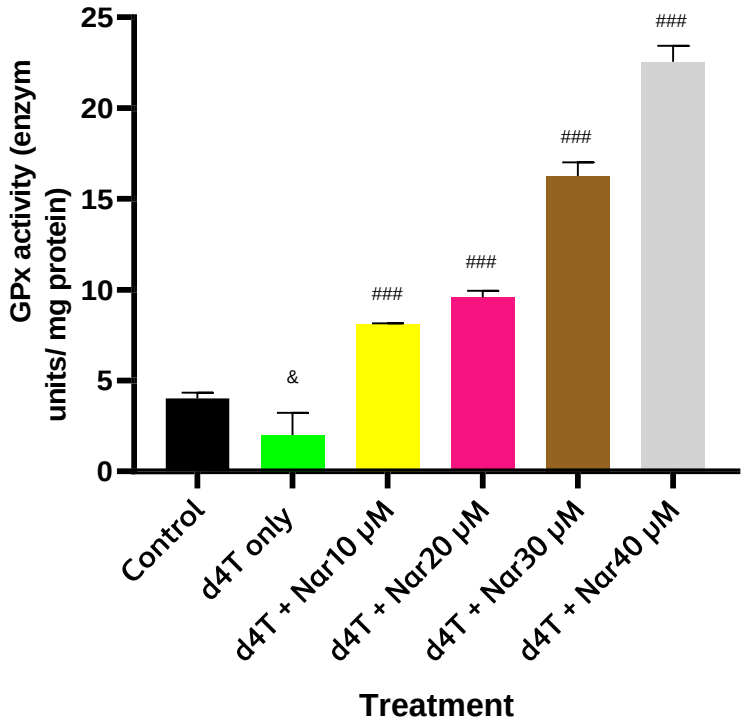


Figure 10: GPx activity in cells treated with d4T and/or different concentrations of naringenin. ($^{\&}$ $p < 0.05$ when compared to control, $^{\#}$ $p < 0.05$ when compared to d4T only.

3.1.4 Cell viability

The cell viability in AZT and d4T exposed cells was significantly ($p < 0.05$) decreased compared to untreated controls (Fig 11, 12). However, cells treated with increasing concentrations of 10-50 µM naringenin had significantly ($p < 0.05$) higher cell viability compared to cells treated with AZT only. Treatment with increasing concentrations of 30 and 40 µM naringenin exhibited significantly ($p < 0.05$) higher cell viability compared to cells treated with d4T only.

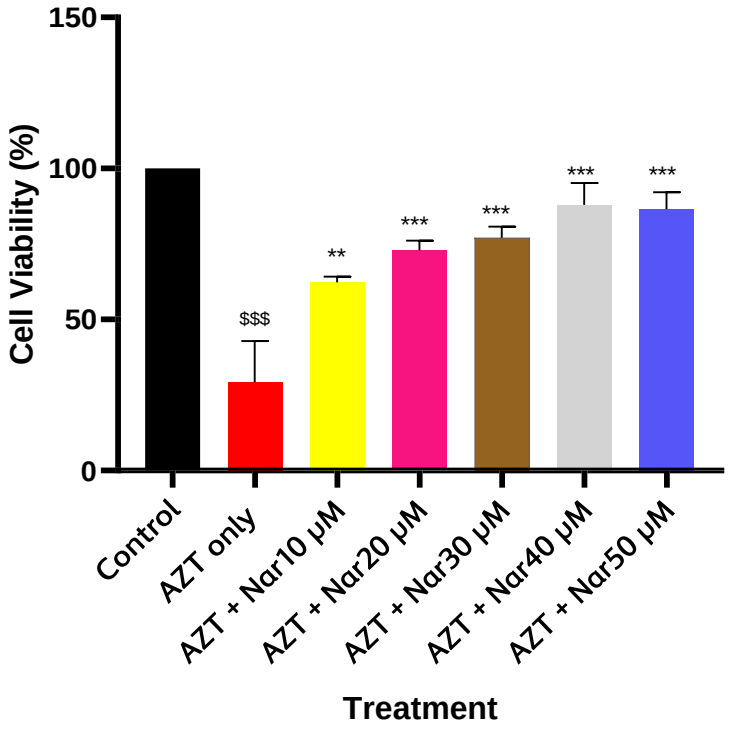


Figure 11: Cell viability in cells treated with AZT and/or different concentrations of naringenin. ($^{\$}p < 0.05$ when compared to control, $*p < 0.05$ when compared to AZT only).

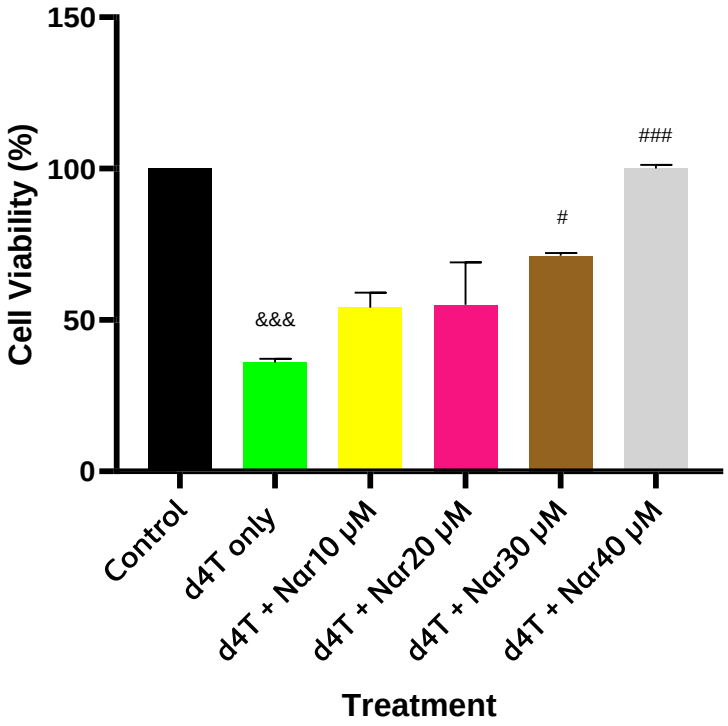


Figure 12: Cell viability in cells treated with d4T and/or different concentrations of naringenin. ($^{\&}$ $p < 0.05$ when compared to control, $^{\#}$ $p < 0.05$ when compared to d4T only)

3.2 Apoptosis Assays

3.2.1 Caspase-3 Assay

Caspase-3 activity significantly ($p < 0.05$) increased in cells treated with AZT and d4T alone compared to the control (Fig 13, 14). Treatment with increasing concentrations of 10 - 50 µM naringenin significantly ($p < 0.05$) reduced caspase-3 activity when compared to cells treated with AZT alone. Treatment with d4T plus increasing concentrations of 20 - 40 µM naringenin showed significantly ($p < 0.05$) reduced caspase-3 activity compared to cells treated with d4T alone.

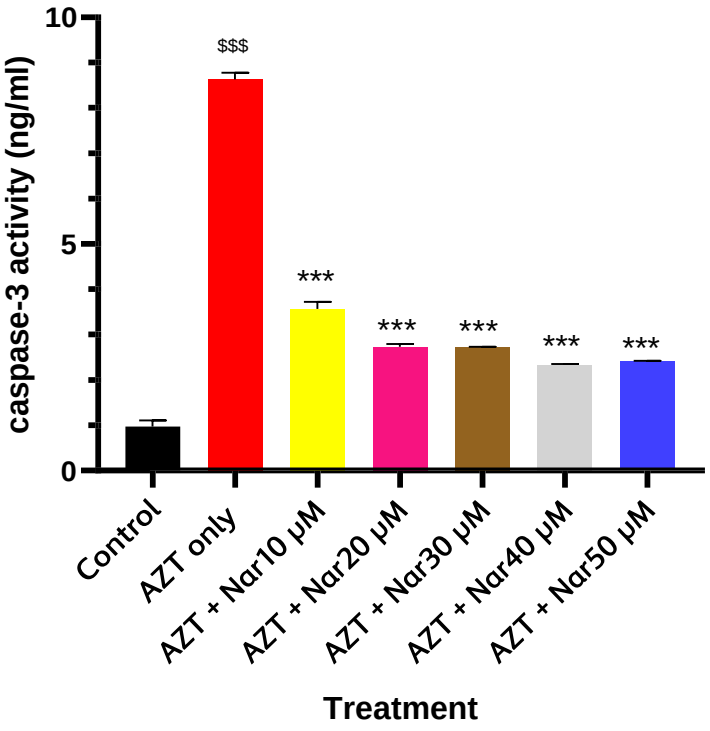


Figure 13: Caspase-3 activity in cells treated with AZT and/or different concentration of naringenin ($^{\$}p < 0.05$ when compared to control, $*p < 0.05$ when compared to AZT only)

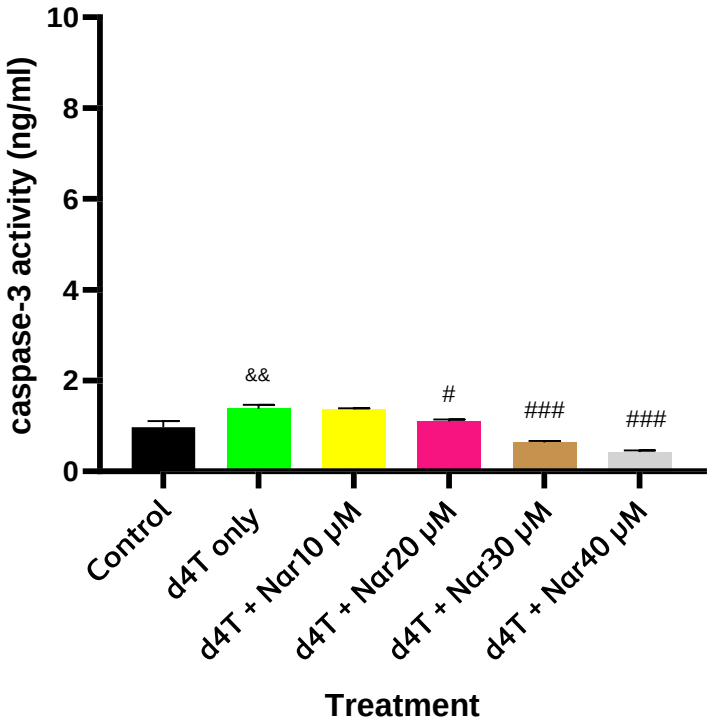


Figure 14: Caspase-3 activity in cells treated with d4T and/or different concentration of naringenin ($^{\&}p < 0.05$ when compared to control, #$p < 0.05$ when compared to d4T only)

3.2.2 Caspase-9 Assay

The caspase-9 activity was significantly ($p < 0.05$) increased in cells treated with NRTIs alone compared to control (Fig 15, 16). Treatment with increasing concentrations of AZT plus 10 - 50 µM naringenin significantly ($p < 0.05$) reduced caspase-9 activity compared to cells treated with AZT alone. Treatment with d4T plus increasing concentrations of 10 - 40 µM naringenin exhibited significant ($p < 0.05$) reduction in caspase-9 activities compared to treatment with d4T alone.

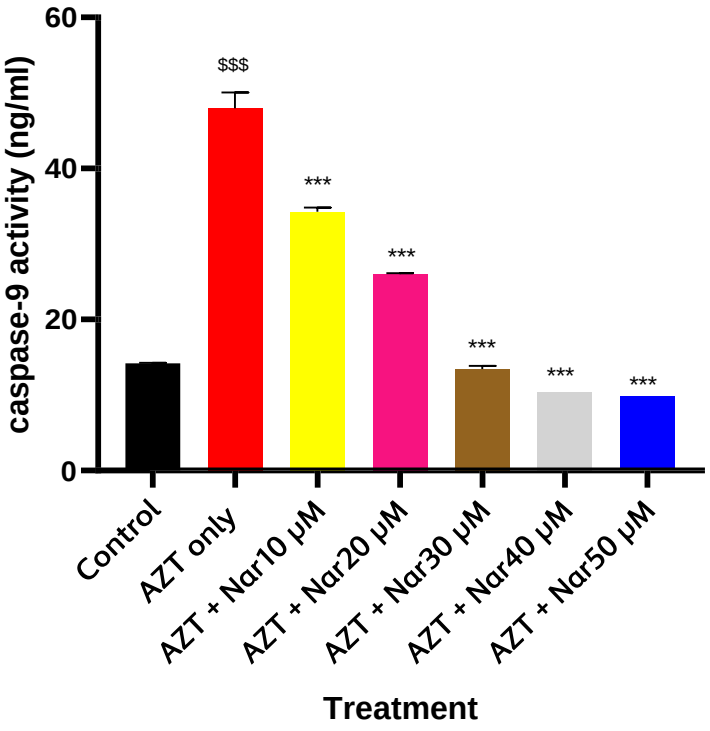


Figure 15: Caspase-9 activity in cells treated with AZT and/or different concentration of naringenin ($^{\$}p < 0.05$ when compared to control, *$p < 0.05$ when compared to AZT only)

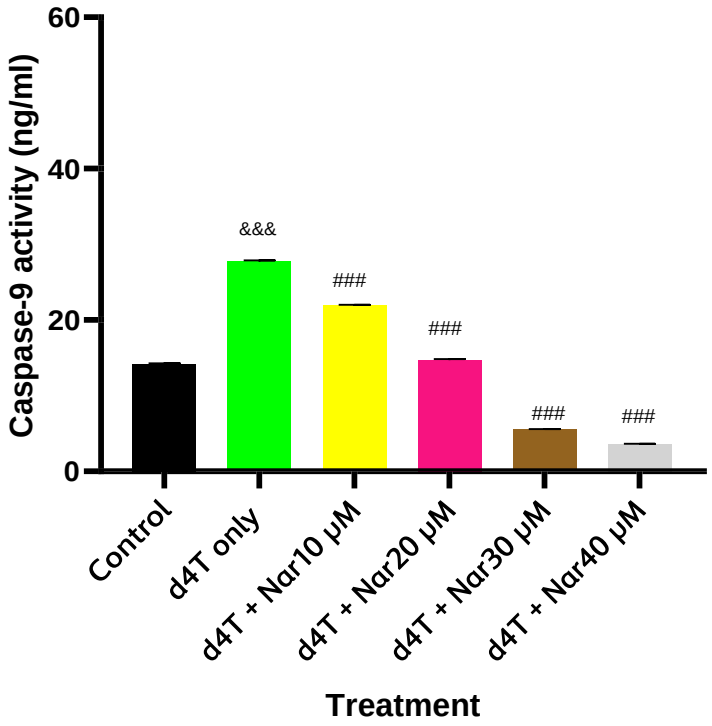


Figure 16: Caspase-9 activity in cells treated with d4T and/or different concentration of naringenin ($^{\&}p < 0.05$ when compared to control, #p < 0.05 when compared to d4T only)

CHAPTER FOUR

4.1 Discussion

NRTI administration has been known to be associated with metabolic disturbances such as cardiomyopathy, neuropathy, myopathies, hyperlactatemia, lactic acidosis, hepatic steatosis, insulin resistance, hepatomegaly and diabetic mellitus [15, 38, 39, 52, 110]. In this study we aimed to elucidate whether naringenin protects against NRTI-induced hepatocyte oxidative stress and apoptosis in Chang cells. Chang cells (CCL-13) are a human cell line derived from human hematoma cells [111]. Chang cells were originally thought to be derived from human liver tissue, however it was later discovered after isoenzyme analysis, that Chang cells in fact had HeLa marker chromosomes possibly due to cell line contamination. Studies however, have reported Chang cells to have similar metabolic functions to normal human liver cells *in vitro* [111, 112]. Therefore, Chang cells were chosen an appropriate hepatocyte model to investigate NRTI-induced oxidative stress and apoptosis in this study. Reduced cell viability, decreased antioxidant activity and increased pro-apoptotic activity were taken as markers of NRTI-induced oxidative stress and apoptosis. The results obtained in this study indicate that increasing concentrations of naringenin significantly reversed NRTI-induced oxidative stress and apoptosis. This was evident by the increase in cell viability and antioxidant enzyme activity as well as the decrease in caspase activity respectively.

Naturally derived phytochemical compounds are increasingly being recognized as promising alternative treatments for various diseases [88, 113]. Naringenin a common dietary flavonoid, has previously been known to possess significant antioxidant and anti-apoptotic properties [80, 104, 114, 115]. It has been suggested that naringenin has potent antioxidant free radical scavenging properties, together with upregulation of antioxidant enzyme activity [79, 81, 94]. In the present study we observed the protective potential of naringenin against NRTI-induced oxidative stress and apoptosis *in vitro*.

In the current study we found that administration of AZT and d4T alone induced overwhelming oxidative stress due to the increased production of ROS consistent with findings by Banajaree et al [116]. Reports have implicated oxidative stress as a key factor in hepatic injury [94, 96]. Oxidative stress is known as the imbalance between the production of ROS and capacity of antioxidant enzymes. This imbalance has formed the basis of various pathological conditions. Overproduction of these highly reactive ROS, bind covalently to cell macromolecules, leading to damage of the cellular framework namely nucleic acid damage, lipid peroxidation and oxidized proteins [45, 96]. Oxidative stress has been known to induce antioxidant defence enzymes [94]. It is well documented that AZT induces oxidative stress in the liver causing damage to and depletion of mitochondrial DNA, possibly due to the mtDNA pol-γ hypothesis [116]. Our research shows that excess ROS is associated with depletion of antioxidant enzyme activity which in turn activates the caspases cascade.

Antioxidant enzymes SOD, catalase, GPx and non-enzymatic antioxidant GSH are essential in order to maintain oxidative balance, therefore changes in their activity can used as biomarkers for oxidative stress [74, 96]. A decrease in the activity of antioxidant enzymatic proteins SOD, catalase and GPx has been consistently noted during oxidative stress [113]. In the present study, the activities of SOD, catalase and GPx were found to be significantly depleted in AZT and d4T alone treatment respectively compared to control which supports evidence that oxidative stress plays a key role in the pathophysiology of NRTI toxicity. Decreased activities of antioxidant enzymes leads to an accumulation of free radicals in hepatocytes, leading to the initiation and propagation of lipid peroxidation which has observed in rats treated with AZT [98, 116]. Administration of increasing concentrations of naringenin in this study however, significantly improved the activities of these antioxidant enzymes thus decreased lipid peroxidation.

The current study found naringenin to effectively neutralize ROS due to the presence of the phenolic structure. This free radical scavenging mechanism prevents oxidative damage thereby forms the basis of naringenin's antioxidant activity [97, 96]. In this study we did not measure ROS concentrations, but cellular content of antioxidant enzymes such as SOD, GPx and CAT were used as acceptable indirect markers of oxidative stress induced by ROS.

SOD is a metalloenzyme known as the first line of defence against ROS, and is responsible for the dismutation of superoxide radicals [74, 95, 107]. Our study shows that treatment with AZT and d4T alone resulted in a decrease of SOD activity compared to control possibly due to the influx of superoxide ions into cellular compartments exhausting SOD activity thus causing oxidative stress (Figures 5 and 6). We found that while AZT and d4T alone triggered oxidative stress, naringenin significantly improved SOD activity in AZT treated cells at 40 and 50 µM concentrations respectively, while in d4T treatment, naringenin significantly improved SOD activity at 10 - 40 µM respectively. Our findings corroborated results from another study which found naringenin to significantly enhance hepatic SOD, CAT, and GPx levels and to reduce the overwhelming production of ROS [93].

Catalase acts as preventative antioxidant and is responsible for transforming harmful hydrogen peroxide produced by SOD to harmless products thus contributing towards better protection against hydrogen peroxide [96]. In line with increased SOD activity, co- treatment with naringenin in AZT treated cells significantly upregulated catalase activity at concentrations 10 - 50 µM respectively (Figure 7). In d4T treatment, naringenin significantly improved catalase activity at 10 - 40 µM respectively (Figure 8), which are in agreement with previous studies [94, 95, 105]. Another study demonstrated that naringenin treatment elevated SOD, GPx and catalase activities consistent with increased gene and protein expressions of these enzymes [74].

Cells treated with AZT and d4T alone exhibited a significant decrease in GPx activity which may be attributed to free radical dependent enzyme deactivation or depletion of co-substrates such as GSH and NADPH. GPx catalyses the reduction of H_2O_2 to H_2O and O_2, utilizing two molecules of GSH to form GSSG [75]. The current study noted that GPx activity following naringenin treatment in AZT treated cells was significantly elevated at concentrations of 40 and 50 µM respectively (Figure 9). Naringenin treatment in d4T treated cells was found to have significantly elevated GPx activity at concentrations of 10 - 40 µM, respectively (Figure 10). Exposure to AZT and d4T treated cells triggered oxidative stress, thus depleting GSH levels and GSH synthesis leading to decreased GPx activity. Thus, naringenin increased gene expression of GPx in order to sufficiently attenuate excess oxidative damage due to hydrogen peroxide. Moreover, flavonoids have shown to modulate the expression of c-glutamylcysteine synthetase, the rate limiting enzyme in the synthesis pathway of GSH [80], thus increasing GSH capacity [117]. Reduction of hydrogen peroxide by GPx occurs in the presence of GSH as a co-enzyme [72]. Therefore, treatment with naringenin restored GSH levels and prevented decrement of GPx activity in AZT and d4T cells, similarly demonstrated by Hermean et al [80]. As shown in the present study, naringenin exhibits therapeutic effects in the early stages of NRTI-induced oxidative stress.

The proposed molecular mechanism by which naringenin counteracts oxidative stress via upregulation of antioxidant enzymes appears to be the Nrf-2 hypothesis. Nrf-2 plays a key role in the transcriptional activation of detoxification and antioxidant proteins [45]. Inactive Nrf2 located in the cytoplasm, interacts with Keap1, becoming active due to the presence of ROS. Free Nrf2 translocate to the nucleus to form a transcriptional complex with Maf family proteins and consequently binds to ARE sequence resulting in coordinated transcriptional activation of antioxidant enzymes [91]. A study by Esmaeli et al reported that treatment with naringenin in a rat model enhanced nuclear expression of Nrf2 which is in accordance with

increased antioxidant enzyme activity observed [94]. In the current study protein expression of Nrf2 was not measured due to resource restraints, however, further investigations should be conducted to determine if increasing concentrations of naringenin induced Nrf-2 activation and attenuated oxidative stress.

Current findings suggest that naringenin, by restoring antioxidant status upon treatment with AZT and d4T possesses potent hepatoprotective properties. Our findings are in line with earlier reports that naringenin significantly improves endogenous serum and antioxidant enzymes biomarkers in a dose-dependent manner and prevents the deleterious effects of oxidative stress [74]. We further showed that increasing concentration of naringenin counteracts oxidative stress at an optimum concentration of 40 - 50 µM. It appears that low concentrations of naringenin exhibited a milder effect seemly to be solely related to the scavenging of ROS, without exerting pro-oxidative properties. At higher concentrations, naringenin significantly increased antioxidant enzyme activity, which suggests that a higher dose of naringenin is required to boost antioxidant enzyme capacity.

Apoptosis can be described as a form of cell death characterized by cell shrinkage, DNA fragmentation, chromatin condensation and formation of apoptotic bodies [105]. Our study found NRTIs to generate ROS, thus triggering the onset of apoptosis. Various studies have reported naringenin to protect against cellular apoptosis [93, 104, 105], therefore we investigated if naringenin alleviated NRTI-induced apoptosis by measuring caspase activity in Chang cells.

Increased oxidative stress results in the depletion of antioxidant enzymes, leading to membrane lipid peroxidation which ultimately triggers extrinsic or intrinsic apoptotic pathways [93, 104]. Caspase-9 and -3 were chosen to measure the extend of apoptosis in this study as they are the rate limiting steps in the apoptosis signalling pathway and are known as

initiator and executioner caspases respectively [102]. Extrinsic pathway for apoptosis can be triggered by the binding of ligands to death receptors which triggers caspases-8, which in turn activates caspse-3, which leads to apoptosis. The intrinsic pathway for apoptosis is activated by ROS, resulting in activation of caspases-9, resulting in the formation of the apoptosome complex (cytochrome c, Apaf-1 and caspase-9). The formation of the apoptosome sequentially activates caspase-3 leading to apoptosis [91, 118]. Once activated, the caspase cascade initiates cell apoptosis leading to cell membrane lysis and secondary necrosis [93]. Our data suggest that AZT and d4T activated caspase-3 and -9 activity, thus inducing hepatocyte apoptosis (Figures 13-16). Preventing or inhibiting caspase activity could therefore possibly prevent hepatocyte damage and hepatotoxicity. Previous studies found that pre-treatment with naringenin significantly inhibited the activation of caspase-3, -8, and -9, therefore preventing hepatocyte apoptosis [93, 106]. The current investigation found that naringenin mitigated caspase-3 and -9 activity and the subsequent caspase cascade in AZT and d4T treated cells. Our reports are supported by a number of previous studies which found naringenin to ameliorate apoptosis [104, 105]. Wang et al suggest that naringenin increases expression of anti-apoptotic factors such as Bcl-2 and down-regulated pro-apoptotic Bax expression thus markedly reducing hepatocyte apoptosis [105].

It is possible to speculate that naringenin due to its antioxidant effects prevents NRTI-induced apoptosis of Chang liver cells and may provide a useful anti-apoptotic intervention for NRTI-induced apoptosis. Kapoor et al reported that naringenin prevented the upregulation of caspase-3 and caspase-9 respectively similar to our study findings [105]. Naringenin therefore prevented ROS production, thus maintained mitochondrial membrane integrity, preventing activation of various apoptogenic factors from mitochondria. Naringenin protected the cells from NRTI-induced caspase-triggered cell death program which ultimately prevented cell necrosis.

We found that the expression of caspase activity correlates with cell viability, as treatment with naringenin at concentrations of 10 - 50 µM significantly improved cell viability in AZT treated cells (Figure 11). Treatment with 30 and 40 µM naringenin respectively was found to enhance cell viability in d4T treated cells (Figure 12). It is interesting to note that d4T had less deleterious effects on cell viability than AZT after 24 h exposure. Moreover, AZT was noted to deplete SOD, GPx activity as well as induce apoptotic enzymes caspase-3 and -9 to a greater extent compared to d4T. This suggests that AZT caused an overproduction of ROS leading to greater exhaustion of cellular antioxidant enzymes.

NRTI –associated pathologies such as lactic acidosis has resulted in withdrawal of drugs such as d4T from first line therapy [41]. While mitochondrion are the targets for NRTI toxicity, mtDNA poly-γ theory is not the sole mechanism by which NRTIs exert their toxic effects [43, 44]. Evidence shows that AZT treatment has no effect on mtDNA [40]. We hypothesize that AZT induces greater oxidative stress (Figures 5, 7, 9) at an acute period compared to d4T which causes chronic oxidative stress resulting in progressive mitochondrial failure. This may be attributed to AZT's chemical structure, which is the only NRTI that possess an azido group which is directly alludes to its pro-oxidant properties. The azido group is lipophilic in nature which enables AZT and its metabolites to target mitochondrial membranes and initiate lipid peroxidation [63, 119]. Nagiah et al further noted that after 24 h exposure to AZT, MDA levels were significantly higher than d4T treated cells [110], similarly reported by Banajaree et al [116], while d4T induced oxidative stress during prolonged exposure (120 h) [110].

While both drugs cause mitochondrial toxicity, chronic use of d4T plays a vital factor in its toxicity as mtDNA decrease continually over time [110]. Our research confirms, that AZT significantly reduced cell viability, depleted antioxidant enzymes and induced caspase activity to a greater extent than d4T after 24h treatment owing to acute oxidative stress rather than chemical cytotoxicity. A study conducted by Kline et al [54] found that acute AZT

exposure in endothelial cells resulted in significant oxidative stress and mitochondrial dysfunction whereas d4T did not [54]. Therefore, AZT and d4T toxicity are time-dependent. It can be concluded that AZT administration resulted in acute oxidative stress, exhausting antioxidant capacity, leading to hepatocyte demise. The spectrum of toxicities differs with different NRTIs. AZT and d4T have different toxicity profiles despite belonging to the same mechanistic class. The concentrations of NRTIs used in this study were chosen to mimic peak plasma concentration when administered at therapeutic doses. Naringenin appears to confer greater protective effects against AZT relative to d4T over 24 h exposure.

AZT's pro-oxidant properties are further attributed to lack of transportation of AZT-MP out of the mitochondrial matrix after formation which leads to continual accumulation of ROS [120]. Nagiah et al found an increase in intracellular ROS after 24 h exposure to AZT [104]. NRTIs cause mitochondrial toxicity due to incorporation with endogenous mtDNA, altering mtDNA pol-γ and mitochondrial activity resulting in mitochondrial depletion [40]. Mitochondrial toxicity may occur due to reduced mitochondrial replication or due to increased degradation of mtDNA. Saitoh A et al postulated that mtDNA dysfunction may be attributed to oxidative stress, uncoupling of proteins, and depletion of deoxyribonucleotide triphosphate pools in mitochondria in addition to mtDNA poly-γ hypothesis [39]. We observed that AZT-induced mitochondrial toxicity is due oxidative stress, similar to findings by an *in vitro* study which showed that AZT increased rather than decreased hepatic mtDNA levels [39, 43], supporting the claim that AZT interacts weakly with mtDNA replication enzyme [62]. 24 h treatment with AZT has shown oxidative damage similar to rats acutely treated with AZT [60]. Therefore, mitochondrion accumulation of ROS by AZT caused mtDNA dysfunction, without involving pol-γ for which it has a low affinity [43]. Another mechanism for AZTs comparatively acute toxicity may be AZT binding to adenyl cylase and inhibiting ADP-ATP translocator. The difference in toxicity profiles of AZT and d4T is due

to differences in pro-drug activation, tissue specificity [60]. In addition, AZT exhibits a slow rate of removal from polymerase-γ as AZT metabolites inhibit exonuclease activity. The accumulation of an increased concentration AZT metabolites in the mitochondria matrix further contribute mitochondrial oxidative stress [121]. AZT-induced inhibition of exonucleases leads to defective proofreading during DNA replication, thus altering mitochondrial gene transcription, leading to mtDNA mutations and impaired OXPHOS [122].

AZT incorporation and chain termination contribute to the production of ROS, leading to mtDNA depletion and loss of OXPHOS [48]. d4T along with ddL and ddC are known to impair mtDNA replication in hepatocytes which in turn induces pro-apoptotic caspase which is evident over prolonged exposure [62]. The severity of antioxidant enzyme depletion upon treatment with AZT and d4T provides a plausible basis for mitochondrial toxicity induced by oxidative stress which supports findings by other studies [54, 110, 116]. We conclude that oxidative stress could be the fundamental pathophysiological pathway which causes damage to cellular machinery in AIDS and its treatment similar to findings by Lewis et al [46].

In addition, naringenin has the ability to reduce cell membrane fluidity due to its lipophilic interaction with polar phospholipids, consequently stabilizing the cell membrane and decreasing interaction of free radicals with mitochondrial membrane thus ameliorating lipid peroxidation due to inhibition of NRTI with mtDNA [71].

Our laboratory has found overwhelming evidence that naringenin ameliorates oxidative stress by boosting antioxidant enzyme defence systems, improving non-enzymatic antioxidant levels such as GSH, directly quenching ROS and reducing pro-apoptotic activity thus reversing apoptosis [21, 38, 45, 107]. We conclude that naringenin can abrogate NRTI-induced hepatotoxicity such as steatosis, lactic acidosis, steatohepatitis and liver failure by abrogating oxidative stress.

4.2 Conclusion

Attenuation and prevention of NRTI-induced adverse effects would improve the efficacy of HIV treatment and improve the quality of life for HIV patients. Our research has highlighted the role of NRTI-induced oxidative stress and apoptosis as well as confirmed the antioxidant and anti-apoptotic capacity of naringenin in hepatocyte protection. There is an urgent need for safe and reliable antioxidant treatment that is capable of reducing oxidative stress-induced damage by NRTIs to improve the clinical outcome of HIV infected patients. Naringenin is a natural, synthetic antioxidant which provides a reasonable therapeutic approach for the prevention and treatment of hepatotoxicity due to NRTI-induced oxidative stress and apoptosis.

Our research provides evidence that naringenin may improve the adverse effects of and adherence to HIV treatment and possibly improve the quality of life of HIV infected patients. Naringenin administration blunted NRTI-associated oxidative stress and apoptosis due to ROS. ROS was either directly quenched by naringenin or neutralized by the increase in endogenous antioxidant enzyme production.

Future investigations should be done to contribute towards establishing and maintaining reasonable therapeutic approaches of international standards which will improve the adverse effects of NRTIs. These studies should look at the mechanisms of NRTI-induced oxidative stress and apoptotic pathways and supplements to mitigate ARV associated side effects.

REFERENCES

1. UNAIDS. *Fact sheet - Latest statistics on the status of the AIDS epidemic, global statistics*.2017. Available from: http://www.unaids.org/en/resources/fact-sheet. [Accessed 24/04/2018]
2. Karim QA. Current status of the HIV epidemic & challenges in prevention. 2017, *Indian J Med Res*. 2017; 146:673-676.
3. UNAIDS. *Country South Africa Overview*. Available from http://www.unaids.org/en/regionscountries/countries/southafrica. [Accessed 24/04/2018]
4. The-Antiretroviral-Therapy-Cohort-Collaboration. Life expectancy of individuals on combination antiretroviral therapy in high-income countries: a collaborative analysis of 14 cohort studies. *Lancet*.2008; 372:293–299.
5. UNAIDS. *Global HIV/AIDS response: epidemic update and health sector progress towards universal access. Progress report 2011*. Available from: http://www.unaids.org/en/media/unaids/contentassets/documents/unaidspublication/2011/20111130_ua_ [Accessed on 24/04/2018].
6. Apostolova N, Blas-Garcia A, Galindo MJ, Esplugues JV. Efavirenz: What is known about the cellular mechanisms responsible for its adverse effects? *Eur J Pharmacol.* 2017; 812:163–173.
7. Fettig J, Swaminathan M, Murrill CS, Kaplan JE. Global Epidemiology of HIV. *Infect Dis Clin N Am*.2014; 28:323–337.
8. Page M, Taylor S. Antiretroviral pharmacology. *MEDICINE*. 2018; 46(5): 288-292.
9. Lv Z, Chu Y, Wang Y. HIV protease inhibitors: A review of molecular selectivity and toxicity. *HIV AIDS (Auckl).* 2015; 7:95-104.
10. Anta L, Llibre JM, Poveda E, Blanco JL, Alvarez M, Perez-Elıas MJ et al. Rilpivirine resistance mutations in HIV patients failing non-nucleoside reverse transcriptase inhibitor-based therapies. *AIDS*. 2013; 27:81–85.
11. Palmer M, Chersich M, Moultrie H, Kuhn L, Fairlie L, Meyers T. Frequency of stavudine substitution due to toxicity in children receiving antiretroviral treatment in Soweto, South Africa. *AIDS*. 2012; 0:1-5.
12. Cihlar T, Ray AS. Nucleoside and nucleotide HIV reverse transcriptase inhibitors: 25 years after zidovudine. *Antiviral Res*. 2010; 85:39–58.

13. Kirby KA, Ryan EM, Sawani AM, Nagy E, Ashida N, Mitsuya H et al. Mechanism of Inhibition of HIV-1 Reverse Transcriptase by 4'-Ethynyl-2-fluoro-2'-deoxyadenosine Triphosphate, a Translocation-defective Reverse Transcriptase Inhibitor. *J Biol Chem*. 2009; 284(51):35681–35691.
14. de Béthune MP. Non-nucleoside reverse transcriptase inhibitors (NNRTIs), their discovery, development, and use in the treatment of HIV-1 infection: A review of the last 20 years (1989–2009). *Antiviral Res*. 2010; 85:75–90.
15. Hoffman RM, Landovitz RJ. Complications of Antiretroviral Therapy for HIV: Mechanisms of Action NRTIs and NNRTIs. *J. Infect. Dis.*.2004; 9(2):1-11.
16. Wu X, Li Y, Peng K, Zhou H. HIV protease inhibitors in gut barrier dysfunction and liver injury. *Curr Opin Pharmacol*. 2014; 19:61–66.
17. Gu WG. Newly approved integrase inhibitors for clinical treatment of AIDS. *Biomed Pharmacother*. 2014; 68:917–921.
18. Wisskirchen K, Lucifora J, Michler T, Protzer U. New pharmacological strategies to fight enveloped viruses. *Trends Pharmacol Sci*. 2014; 35(9):470-478.
19. Andrade CH, de Freitas LM, de Oliveira V. Twenty-six years of HIV science: an overview of anti-HIV drugs metabolism. *Braz. J. Pharm. Sci*. 2011; 47:209-230.
20. Gardner K, Hall PA, Chinnery PF, Payne BA. HIV treatment and associated mitochondrial pathology: review of 25 years of in vitro, animal and human studies. *Toxicol Pathol*. 2014; 42(5):811-822.
21. Nzuza S, Ndwande DE, Owira PM. Naringin protects against HIV-1 protease inhibitors-induced pancreatic B-cell dysfunction and apoptosis. *Mol Cell Endocrinol.* 2016; 437:1-10.
22. Palella FJ Jr, Delaney KM, Moorman AC, Loveless MO, Fuhrer J, Satten GA, Aschman DJ, Holmberg SD. Declining morbidity and mortality among patients with advanced human immunodeficiency virus infection. HIV outpatient study investigators. *N Engl J Med*.1998; 338:853–60.
23. Chang CY, Schiano TD. Review article: drug hepatotoxicity. Aliment *Pharmacol Ther*. 2007; 25:1135–1151.
24. Kaspar MB, Sterling RK. Mechanism of liver disease in patients infected with HIV. *BMJ Open Gastro*. 2017; 4:1-8.
25. Smith C, Sabin CA, Lundgren JD, Thiebaut R, Weber R, Law M, et al. Factors associated with specific causes of death amongst HIV-positive individuals in the D: A: D Study. *AIDS.* 2010; 24: 1537-1548.

26. Belloso WH, Orellana LC, Grinsztejn B, Madero JS, La Rosa A, Veloso VG, et al. Analysis of serious non-AIDS events among HIV-infected adults at Latin American sites. *HIV Med*. 2010; 11: 554-564.
27. Kovari H, Ledergerber B, Battegay M, Rauch A, Hirschel B, Foguena AK, et al. Incidence and risk factors for chronic elevation of alanine aminotransferase levels in HIV-infected persons without hepatitis b or c virus co-infection. *Clin Infect Dis*. 2010; 50: 502-511.
28. Azua OO, Jegedea AI, Ugochukwua O, Onanugaa IO, Kharwaa S, Naidu EC. Hepatic histomorphological and biochemical changes following highly active antiretroviral therapy in an experimental animal model: Does *Hypoxis hemerocallidea* exacerbate hepatic injury? *Toxicol Rep*. 2016; 3:114-112.
29. Flexner C. HIV-Protease inhibitors. *N Engl J Med*.1998; 338:1281-92.
30. Sulkowski MS. Drug-induced liver injury associated with ARV therapy that includes HIV-1 Protease inhibitors. *Clin Infect Dis*. 2004; 38(2):90–7.
31. Dieterich DT, Robinson PA, Love J, Stern JO. Drug-Induced Liver Injury Associated with the Use of Non-Nucleoside Reverse-Transcriptase Inhibitors. *Clin Infect Dis*. 2004; 38(2):80–9.
32. Arauz J, Ramos-Tovar E, Muriel P. Redox state and methods to evaluate oxidative stress in liver damage: From bench to bedside. *Ann. Hepatol*. 2016; 15(2): 160-173.
33. Williams K, Rao YP, Natarajan R, Pandak WM, Hylemon PB. Indinavir alters sterol and fatty acid homeostatic mechanisms in primary rat hepatocytes by increasing levels of activated sterol regulatory element-binding proteins and decreasing cholesterol 7alpha-hydroxylase mRNA levels. *Biochem. Pharmacol*. 2004; 67:255–67.
34. van Griensven J, Zachariah R, Rasschaert F, Mugabo J, Attéa EF, Reid T. Stavudine- and nevirapine-related drug toxicity while on generic fixed-dose antiretroviral treatment: incidence, timing and risk factors in a three-year cohort in Kigali, Rwanda. *Transactions of the Royal Society of Tropical Medicine and Hygiene*. 2009; 1-6.
35. Fortuin-de Smidt M, de Waal R, Cohen K, Technau K, Stinson K, Maartens G, et al. First-line antiretroviral drug discontinuations in children. *PLoS One*. 2017; 12(2): 1-9.
36. Nunez M. Hepatotoxicity of antiretrovirals: Incidence, mechanisms and management. *J Hepatol*. 2006; 44:S132–S139.
37. Wambani JR, Ogola PE, Arika WM, Rachuonyo HO, Kemboi NG, Lihana R, et al. Anti-Retroviral Drug Hepatotoxicity and Risk Factors in HIV Patients with or

Without Hepatitis B and C: A Review. *Infect Dis Ther.* 2015; 3(6): 332-0877. DOI: 10.4172/2332-0877.1000258.

38. Adebiyi O, Adebiyi OA, Owira, PMO. Naringin Reverses hepatocyte apoptosis and oxidative stress associated with HIV-1 Nucleotide Reverse Transcriptase Inhibitors-induced metabolic complications. *Nutrients*. 2015; 7(12):10352–10368.
39. Saitoh A, Haas RH, Naviaux RK, Salva NG, Wong JK, Spector SA. Impact of Nucleoside Reverse Transcriptase Inhibitors on Mitochondrial DNA and RNA in Human Skeletal Muscle Cells. *Antimicrob Agents Chemother*. 2008; 8(52):2825–2830.
40. Vinogradov SV, Poluektova LY, Makarov E, Gerson T, Senanayake MT. Nano-NRTIs: efficient inhibitors of HIV type-1 in macrophages with a reduced mitochondrial toxicity. *Antivir Chem Chemother*. 2010; 21:1–14.
41. Eakins J, Bauch C, Woodhouse H, Park B, Bevan S, Dilworth C, Walker P. A combined in vitro approach to improve the prediction of mitochondrial toxicants. *Toxicol in Vitro*. 2016; 34:161–170.
42. Gil L, Pérez D, Tápanes R, Pérez J, Grune T. Does mitochondrial dysfunction during antiretroviral therapy in human immunodeficiency virus infection suggest antioxidant supplementation as a beneficial option? *Redox Rep*. 2013; 10 (3): 113-119. DOI: 10.1179/135100005X38905.
43. Lund KC, Peterson LL, Wallace KB. Absence of a Universal Mechanism of Mitochondrial Toxicity by Nucleoside Analogs. *Antimicrob Agents Chemother*. 2007; 51(7): 2531–2539.
44. Ho¨schele D. Cell culture models for the investigation of NRTI-induced mitochondrial toxicity. Relevance for the prediction of clinical toxicity. *Toxicol in Vitro*. 2006; 20:535–546.
45. Adebiyi O, Adebiyi O, Owira P. Naringin ameliorates HIV-1 Nucleoside Reverse Transcriptase Inhibitors-induced mitochondrial toxicity. *Curr. HIV Res*. 2016; 14(5):1-11.
46. Lewis W. Mitochondrial dysfunction and nucleoside reverse transcriptase inhibitor therapy: experimental clarifications and persistent clinical questions. *Antiviral Res*. 2003;58(3):189-197.
47. Kohler JJ, Lewis W. A Brief Overview of Mechanisms of Mitochondrial Toxicity From NRTIs. *Environ Mol Mutagen*. 2007; 48:166-172.

48. Burkus G, Hitchcock MJM, Chilar T. Mitochondrial toxicity of NRTIs: in vitro assessment and comparison with tenofovir. *Antimicrob Agents Chemother*. 2002; 46(3):716-23.
49. Nagiah S, Phulukdaree A, Chuturgoon AA. Lon protease and eiF2a are involved in acute, but not prolonged, antiretroviral induced stress response in HepG2 cells. *Chem Biol Interac*. 2016; 252: 82-86.
50. Joshi D, O'Grady J, Dieterich D, Gazzard B, Agarwal K. Increasing burden of liver disease in patients with HIV infection. *Lancet*. 2011; 377: 1198–1209.
51. Nolan D, Hammond E, Martin A, Taylor L, Herrmann S, McKinnon E, Metcalf C, Lathama B, Mallal S. Mitochondrial DNA depletion and morphologic changes in adipocytes associated with nucleoside reverse transcriptase inhibitor therapy. *AIDS* 2003, 17:1329–1338.
52. Setzer B, Lebrecht D, Walker UA. Pyrimidine Nucleoside Depletion Sensitizes to the Mitochondrial Hepatotoxicity of the Reverse Transcriptase Inhibitor Stavudine. *Am J Pathol*.2008; 172(3):681-690.
53. Lutu MR, Nzuza S, Mato PEM, Govender K, Mphumelelo Gumede LM, Kumalo, et al. Image taken from DNA polymerase-γ hypothesis in nucleoside reverse transcriptase-induced mitochondrial toxicity revisited: A potentially protective role for citrus fruit derived naringenin? *Eur. J. Pharmacol.* 2019; 852:159-166.
54. Kline ER, Bassit L, Hernandez-Santiago BI, Detorio MA, Liang B, Kleinhenz DJ, et al. Long-Term Exposure to AZT, but not d4T, Increases Endothelial Cell Oxidative Stress and Mitochondrial Dysfunction. *Cardiovasc. Toxicol*. 2009; 9(1): 1–12. doi:10.1007/s12012-008-9029-8.
55. Johnson AA, Ray AS, Hanes J, Suo Z, Colacino JM, Anderson KS, et al. Toxicity of Antiviral Nucleoside Analogs and the Human Mitochondrial DNA Polymerase. *J Biol Chem.* 2001; 276(44):40847–40857.
56. Mason PA and Cox LS. The role of DNA exonucleases in protecting genome stability and their impact on ageing. *AGE*. 2012; 34:1317–1340.
57. Hanes JW and Johnson KA. Exonuclease Removal of Dideoxycytidine (Zalcitabine) by the Human Mitochondrial DNA Polymerase. *Antimicrob Agents Chemother.* 2008; 52(1): 253–258.
58. Cooper DL and Lovett ST. Toxicity and tolerance mechanisms for azidothymidine, a replication gap-promoting agent, in Escherichia coli. *DNA Repair (Amst*). 2011; 10(3): 260–270.

59. Lim, S. E. & Copeland, W. C. Differential incorporation and removal of antiviral deoxynucleotides by human DNA polymerase . *J. Biol. Chem.* 276, 23616-23623.
60. Szabados E, Fischer G.M, Toth K, Csete B, Nemeti B, Trombitas K, et al. Role of reactive oxygen species and poly-ADP-ribose polymerase in the development of AZT-induced cardiomyopathy in rat. *Free Radic. Biol. Med*. 1999; 26 (3-4): 309–317.
61. Kakuda TN. Pharmacology of nucleoside and nucleotide reverse transcriptase inhibitor-induced mitochondrial toxicity. *Clin. Ther*. 2000; 22(6):346-355.
62. Venoff N, Setzer B, Melkaoui K, Walker UA. Mitochondral toxicity of tenofovir emtricitabine and abacair alone and in combination with additional nucleoside reverse transcriptase inhibitors. *Antivir. ther*. 2007; 12(7):075-1085.
63. Komarov AM, Hall JM, Weglicki WB. Azidothymidine promotes free radical generation by activated macrophages and hydrogen peroxide-iron-mediated oxidation in a cell-free system. *Biochimica et Biophysica Acta* 2004;1688(3):257–264.
64. Côté HCF. Possible ways nucleoside analogues can affect mitochondrial DNA content and gene expression during HIV therapy. *Antivir. Ther.* 2005; 10(Sup1):83-91.
65. Bannister WP, Friis –Moller N, Mocroft A, Viard JP, van Lunzen J, Kirk O, et al. Incidence of abacavir hypersensitivity in EuroSIDA. *Antivir. Ther.* 2008; 13:687-696.
66. Sousa-Pinto B, Pinto-Ramos J, Correia C, Goncalves-Costa G, Gomes L, Gil-Mata S, et al. Pharmacogenetics of abacavir hypersensitivity: A systematic review and meta-analysis of the association with HLA-B*57:01. *J. Allergy Clin. Immunol.* 2015; 136(4): 1092-1094
67. Tozzi V. Pharmacogenetics of antiretrovirals. *Antiviral Res*. 2010; 85:190–200.
68. Poljsak B, Šuput D, Milisav I. Achieving the Balance between ROS and Antioxidants: When to Use the Synthetic Antioxidants. *Oxid Med Cell Longev*. 2013: 1-13.
69. Sharma B. Oxidative Stress in HIV Patients Receiving Antiretroviral Therapy. *Curr. HIV Res.* 2014; 12:13-21.
70. Cao L, Quan X,Zeng WJ, Yang XO, Wang MJ. Mechanism of Hepatocyte Apoptosis. *J Cell Death*. 2016; 9:19-29.
71. Li S, Tan H, Wang N, Zhang Z, Lao L, Wong C, Feng Y. The Role of Oxidative Stress and Antioxidants in Liver Diseases. *Int. J. Mol. Sci.* 2015; 16:26087–26124.
72. Ivanov AV, Valuev-Elliston VT, Ivanova ON, Kochetkov SN, Starodubova ES, Birke B, et al. Oxidative Stress during HIV Infection: Mechanisms and Consequences. *Oxid Med Cell Longev*. 2016:1-18.

73. Lazo-de-la-Vega-Monroy ML, Fernández-Mejía C. Image from Oxidative Stress in Diabetes Mellitus and the Role Of Vitamins with Antioxidant Actions. 2013; 209-232.
74. Mershiba SD, Dassprakash MV, Saraswathy SD. Protective effect of naringenin on hepatic and renal dysfunction and oxidative stress in arsenic intoxicated rats. *Mol Biol Rep*. 2013; 40:3681–3691.
75. Hernández-Aquino E, Zarco N, Casas-Grajales S, Ramos-Tovar E, Flores-Beltrán RE, Arauz J et al. Naringenin prevents experimental liver fibrosis by blocking TGFβ-Smad3 and JNK-Smad3 pathways. *World J Gastroenterol*. 2017; 23(24): 4354-4368.
76. Kao E, Shinohara M, Feng M, Lau MY, Ji C. Human immunodeficiency virus protease inhibitors modulate Ca2+ homeostasis and potentiate alcoholic stress and injury in mice and primary mouse and human hepatocytes. *Hepatology*. 2012; 56:594–604.
77. Soriano V, Puoti M, Garcia-Garsco P, Rockstroh JK, Benhamou Y, Barreiro P, et al. Antiretroviral drugs and liver injury. *AIDS*. 2008; 22:1–13.
78. Manzo-Avalos S, Saavedra-Molina A. Cellular and mitochondrial effects of alcohol consumption. *Int J Environ Res Public Health*. 2010; 7:4281–304.
79. Adil M, Kandhare AD, Ghosh P, Venkata S, Raygude KS, Bodhankar SL. Ameliorative effect of naringin in acetaminophen induced hepatic and renal toxicity in laboratory rats: role of FXR and KIM-1. *Ren Fail*. 2016; 38(6):1007-1020, DOI: 10.3109/0886022X.2016.1163998.
80. Hermenean A, Ardelean A, Stan M, Hadaruga N, Mihali CV, Marieta Costache, Dinischiotu A. Antioxidant and Hepatoprotective Effects of Naringenin and Its b-Cyclodextrin Formulation in Mice Intoxicated with Carbon Tetrachloride: A Comparative Study. *J. Med. Food*. 2013; 00(0):1-8.
81. Chen R, Qi QL, Wang M, Li Q. Therapeutic potential of naringin: an overview. *Pharm. Biol*. 2016; 54(12):3203-3210.
82. Han Y, Su J, Liu X, Zhao Y, Wang C, Li X. Naringin alleviates early brain injury after experimental subarachnoid hemorrhage by reducing oxidative stress and inhibiting apoptosis. *Brain Res Bull*. 2017; 133:42–50.
83. Al-Harbi MS. Hepatoprotective effect and antioxidant capacity of naringenin on arsenic-induced liver injury in rats. *Int J Pharm Pharm Sci.* 2016; 8(4):103-108.
84. Dong D, Xu L, Yin L,Qi Y, Peng J. Naringin prevents carbon tetrachloride-induced acute liver injury in mice. *J Funct Foods*. 2015; 12:179–191.

85. El-desokya AH, Abdel-Rahmanb RF, Ahmed OK, El-Beltagic HK, Hattorid M. Anti-inflammatory and antioxidant activities of naringin isolated from Carissa carandas L.: In vitro and in vivo evidence. P*hytomedicine*. 2018: 42:126–134.
86. Gasparrini M, Giampieri F, Alvarez Suarez JMA, Mazzoni L, Hernandez TYF, Quiles JL, et al. Image from AMPK as a New Attractive Therapeutic Target for Disease Prevention: The Role of Dietary Compounds AMPK and Disease Prevention. *Curr Drug Targets*. 2016; 17:865-889.
87. Karim N, Jiac Z, Zhenga X, Cuib S,Chena W. A recent review of citrus flavanone naringenin on metabolic diseases and its potential sources for high yield-production. *Trends Food Sci Technol*. 2018; 79:35–54.
88. Kanaze FI, Bounartzi MI, Georgarakis M, Niopas I. Pharmacokinetics of the citrus flavanone aglycones hesperetin and naringenin after single oral administration in human subjects. *Eur J Clin Nutr*. 2007; 61:472–477.
89. Bharti S, Rani N, Krishnamurthy B, Arya DS. Preclinical Evidence for the Pharmacological Actions of Naringin: A Review. *Planta Med*. 2014; 80: 437– 451.
90. Chabane MN, Ahmad AA, Peluso J, Muller CD, Ubeaud G. Quercetin and naringenin transport across human intestinal Caco-2 cells. *JPP*. 2009; 61: 1473–1483.
91. Hernández-Aquino E, Muriel P. Beneficial effects of naringenin in liver diseases: Molecular mechanisms. *World J Gastroenterol*. 2018; 24(16): 1679–1707.
92. Shulman M, Cohen M, Soto-Gutierrez A, Yagi H, Wang H, Goldwasser J, Lee-Parsons CW, Benny-Ratsaby O, Yarmush ML, Nahmias Y. Enhancement of Naringenin Bioavailability by Complexation with Hydroxypropoyl-β-Cyclodextrin. *PLoS One*. 2011; 6(4): e18033.
93. Yen FL, Wu TH, Lin LT, Cham TM, Lin CC. Naringenin-Loaded Nanoparticles Improve the Physicochemical Properties and the Hepatoprotective Effects of Naringenin in Orally-Administered Rats with CCl4-Induced Acute Liver Failure. *Pharm. Res*. 2009; 26 (4):893-902. DOI: 10.1007/s11095-008-9791-0.
94. Esmaeili MA, Alilou M. Naringenin attenuates CCl4-induced hepatic inflammation by the activation of an Nrf2-mediated pathway in rats. *Clin Exp Pharmacol Physiol*. 2014; 41:416–422.
95. Jayaraman J, Veerappan M, Namasivayam N. Potential beneficial effect of naringenin on lipid peroxidation and antioxidant status in rats with ethanol-induced hepatotoxicity. *JPP*. 2009; 61:1383–1390.

96. Miler M, Zivanovic J, Ajdzanovic V, Orescanin-Dusic Z, Milenkovic D, Konic-Ristic A, Blagojevic D, Milosevic V, Sosic-Jurjevic B. Citrus flavanones naringenin and hesperetin improve antioxidant status and membrane lipid compositions in the liver of old-aged Wistar rats. *Exp Gerontol*. 2016; 84:49-60.
97. Arora A, Byrem TM, Nair MG, Strasburg GM. Modulation of Liposomal Membrane Fluidity by Flavonoids and Isoflavonoids. *Arch Biochem Biophys*. 2000; 373 (1):102–109.
98. Pari L, Gnanasoundari M. Influence of Naringenin on Oxytetracycline Mediated Oxidative Damage in Rat Liver. *Basic Clin Pharmacol Toxicol*. 2006; 98:456–461.
99. Casas-Grajales S, Muriel P. Antioxidants in liver health. *World J Gastrointest Pharmacol Ther*. 2015; 6(3):59-72.
100. Alam MA, Subhan N, Rahman MM, Uddin SJ, Reza HM, Sarker SD. Effect of Citrus Flavonoids, Naringin and Naringenin, on Metabolic Syndrome and Their Mechanisms of Action. *Adv. Nutr*. 2014; 5: 404–417.
101. Orrenius S. Apoptosis : molecular mechanisms and implications for human disease. *J. Intern. Med*. 1995; 237:529-536
102. Elmore S. Apoptosis: A Review of Programmed Cell Death. *Toxicol Pathol*. 2007; 35(4):495–516.
103. Brenner C, Galluzzi L, Kepp O, Kroemer G. Decoding cell death signals in liver inflammation. *J Hepatol*. 2013; 59:583–594.
104. Al-Dosari DI, Ahmed MM, Al-Rejaie SS, Alhomid AS, Ola MS. Flavonoid Naringenin Attenuates Oxidative Stress, Apoptosis and Improves Neurotrophic Effects in the Diabetic Rat Retina. *Nutrients*. 2017; 9:(1161) 1-13.
105. Kapoor R, Kakkar P. Naringenin accords hepatoprotection from streptozotocin induced diabetes in vivo by modulating mitochondrial dysfunction and apoptotic signaling cascade. *Toxicology Reports*. 2014; 1:569–581.
106. Wang C, Fan RQ, Zhang YX, Nie H, Li K. Naringenin protects against isoniazid- and rifampicin induced apoptosis in hepatic injury. *World J Gastroenterol*. 2016; 22(44):9775-9783.
107. Nzuza S, Zondi S, Owira. PMO Naringin prevents HIV-1 protease inhibitors induced metabolic complications in vivo. *PLoS ONE*. 2017; 12(11): e0183355. https://doi.org/10.1371/journal.pone.0183355.
108. Gil L, Pérez D, Tápanes R, Pérez J, Grune T. Does mitochondrial dysfunction during antiretroviral therapy in human immunodeficiency virus infection suggest

antioxidant supplementation as a beneficial option? *Redox Report.* 2013; 10 (3): 113-119. DOI: 10.1179/135100005X38905.

109. Fautz R, Husein B, Hechenberger C. Application of the neutral red assay (NR assay) to monolayer cultures of primary hepatocytes: rapid colorimetric viability determination for the unscheduled DNA synthesis test (UDS). *Mutat.Res*.1991; 253(2):173-179.

110. Nagiah S, Phulukdaree A, Chuturgoon A. Mitochondrial and Oxidative Stress Response in HepG2 Cells Following Acute and Prolonged Exposure to Antiretroviral Drugs. *J Cell Biochem*. 2015; 116:1939–1946.

111. Yang T, Li C, Zhang L, Li M, Zhou P. Promising Hepatocyte-Like Cell Line, CCL-13, Exhibits Good Liver Function Both In Vitro and in an Acute Liver Failure Model. *Transplant Proc*. 2013; 45:688–694.

112. Wang G, Gong Y, Anderson J, Sun D, Minuk G, Roberts MS, Burczynski FJ. Antioxidative Function of L-FABP in L-FABP Stably Transfected Chang Liver Cells. *Hepatology*. 2005; 42(4):871-879.

113. Ali R, Shahid A,Ali N, Hasan SK, Majed F, Sultana S. Amelioration of Benzo[a]pyreneinduced oxidative stress and pulmonary toxicity by Naringenin in Wistar rats: A plausible role of COX-2 and NF-κB. *Hum Exp Toxicol*. 2017; 36(4):349–364.

114. Takashi Isobe, Susumu Ohkawara, Sadayuki Ochi, Toshiko Tanaka-Kagawa, Hideto Jinno, Nobumitsu Hanioka. Naringenin glucuronidation in liver and intestine microsomes of humans, monkeys, rats, and mice. *Food Chem Toxicol*. 2018; 111:417–422.

115. Lin H, Ku K, Lin I, Yeh. Naringenin attenuates hepatitis B virus X protein- induced hepatic steatosis. *BMC Complement Altern Med*. 2017; 17:505

116. Banerjee A, Abdelmegeed MA, Jang S, Song BJ. Zidovudine (AZT) and Hepatic Lipid Accumulation: Implication of Inflammation, Oxidative and Endoplasmic Reticulum Stress Mediators. *PLOS ONE*. 2013; 8(10): 1-9.

117. Manchope MF, Casagrande R, Verri, Jr. WA. Naringenin: an analgesic and anti-inflammatory citrus flavanone. *Oncotarge*t. 2017; 8(3): 3766-3767.

118. Eguchi A, Wree, A Feldstein AE. Biomarkers of liver cell death. *J Hepatol*. 2014: 60:1063–1074.

119. Mak IT, Nedelec LF, Weglicki WB. Pro-oxidant properties and cytotoxicity of AZT-monophosphate and AZT. *Cardiovasc. Toxicol*. 2004;4(2):109–115.

120. McKee EE, Bentley AT, Hatch M, Gingerich J, Susan-Resiga D. Phosphorylation of thymidine and AZT in heart mitochondria: Elucidation of a novel mechanism of AZT cardiotoxicity. *Cardiovasc. Toxicol*. 2004;4(2):155–167.
121. Pan-Zhou XR,Cui L, Zhou XJ, Sommadossi JP, Darley-Usmar VM. Differential Effects of Antiretroviral Nucleoside Analogs on Mitochondrial Function in HepG2 Cells. *Antimicrob Agents Chemother*. 2000; 44(3):496–503.
122. Harrington JA, Reardon JE, Spector T. 3'-Azido-3'-Deoxythymidine (AZT) Monophosphate: An Inhibitor of Exonucleolytic Repair of AZT-Terminated DNA. *Antimicrob Agents Chemother*. 1993; 37(4):918-920.

APPENDICIES

Appendix 1: Publication, 'DNA polymerase-γ hypothesis in nucleoside reverse transcriptase-induced mitochondrial toxicity revisited: A potentially protective role for citrus fruit derived naringenin?'.

European Journal of Pharmacology 852 (2019) 159–166

Contents lists available at ScienceDirect

European Journal of Pharmacology

journal homepage: www.elsevier.com/locate/ejphar

DNA polymerase-γ hypothesis in nucleoside reverse transcriptase-induced mitochondrial toxicity revisited: A potentially protective role for citrus fruit-derived naringenin?

Mathabo Ruth Lutu, Sanelisiwe Nzuza, Pascale Edith Mofo Mato, Khmera Govender, Londiwe Mphumelelo Gumede, Sinenhlanhla Innocentia Kumalo, Nontokozo Nomfundo Mlambo, Ranjendraparsad Hurchund, Peter Mark Oroma Owira*

Molecular and Clinical Pharmacology Research Laboratory, Department of Pharmacology, Discipline of Pharmaceutical, School of Health Sciences, University of KwaZulu- Natal, Westville Campus, Private Bag X5400, Durban 3629, South Africa

ARTICLE INFO

Keywords:
NRTIs
Mitochondria
Naringenin
DNA polymerase

ABSTRACT

Nucleoside reverse transcriptase inhibitors (NRTIs) form the backbone in combination antiretroviral therapy (cARVs). They halt chain elongation of the viral cDNA by acting as false substrates in counterfeit incorporation mechanism to viral RNA-dependent DNA polymerase. In the process genomic DNA polymerase as well as mitochondrial DNA (mtDNA) polymerase-γ (which has a much higher affinity for these drugs at therapeutic doses) are also impaired. This leads to mitochondrial toxicity that manifests clinically as mitochondrial myopathy, peripheral neuropathy, hyperlactatemia or lactic acidosis and lipoatrophy. This has led to the revision of clinical guidelines by World Health Organization to remove stavudine from first-line listing in the treatment of HIV infections. Recent reports have implicated oxidative stress besides mtDNA polymerase-γ hypothesis in NRTI-induced metabolic complications. Reduced plasma antioxidant concentrations have been reported in HIV po- sitive patients on cARVs but clinical intervention with antioxidant supplements have not been successful either due to low efficacy or poor experimental designs.

Citrus fruit-derived naringenin has previously been demonstrated to possess antioxidant and free radical scavenging properties which could prevent mitochondrial toxicity associated with these drugs. This review re- visits the controversy surrounding mtDNA polymerase-γ hypothesis and evaluates the potential benefits of naringenin as a potent anti-oxidant and free radical scavenger which as a nutritional supplement or therapeutic adjunct could mitigate the development of mitochondrial toxicity associated with these drugs.

1. Introduction

Human immunodeficiency virus (HIV) infections cause Acquired Immune-Deficiency Syndrome (AIDS) which has remained a serious global health problem since the epidemic began in 1983. Close to 1/3 of the approximately 60 million people infected with HIV globally have since died (Maartens et al., 2014; UNAIDS, 2016). Combination Antiretroviral Therapy (cARV) was introduced to reduce morbidity and mortality by increasing the CD4+ cell counts and reduce viral loads and prevent opportunistic infections that cause AIDS (Anuurad et al., 2010; Bekker et al., 2014). Current World Health Organization (WHO) and international guidelines for the treatment of HIV infections re- commend cARV containing any 2 of Nucleoside/Nucleotide Reverse Transcriptase Inhibitors (NRTIs) as a backbone, and any 1 of Non-Nucleoside Reverse Transcriptase (NNRTIs) or a Protease Inhibitor (PI), (ritonavir-boosted) or integrase/fusion (entry) inhibitor as a base, depending on safety, tolerability and efficacy (Aid for Aids, 2016; Gardner et al., 2014; World Health Organisation, 2002), (Table 1). NRTIs act as false substrates to HIV-1 reverse transcriptase which lack an essential motif, a 3'-hydroxyl group which after incorporation into a growing cDNA cannot mount nucleophilic attack on the incoming dNTP. This leads to abortion of proviral cDNA chain elongation process (Apostolova et al., 2011; Barbaro, 2006; El Safadi et al., 2007) (Fig. 1). NRTIs are initially activated intracellularly by tri-phosphorylation (except tenofovir, which already has an attached phosphate group, hence is classified as nucleotide and only undergoes bi-phosphorylation) (Fig. 1). In case of AZT, the second phosphorylation step is the rate-limiting one while tenofovir disoproxil fumerate (TDF) is already

* Corresponding author.
E-mail address: owirap@ukzn.ac.za (P.M. Oroma Owira).

https://doi.org/10.1016/j.ejphar.2019.03.017
Received 11 December 2018; Received in revised form 11 March 2019; Accepted 11 March 2019
Available online 12 March 2019
0014-2999/

Table 1
Antiretroviral drugs, their mechanisms of action and common side-effects. Where side-effects are specific to class components are indicated.

Class drugs agents	Antiretroviral	Mechanism of action	Common side-effects
Nucleoside/nucleotide reverse transcriptase inhibitors (NRTI)	Abacavir (ABC) Didanosine (ddI)	Counterfeit incorporation which terminates viral cDNA chain elongation	Lipoatrophy (d4T, AZT) hyperlactatemia (d4T, ddI, AZT), peripheral neuropathy (d4T, AZT, ddI), steatohepatitis (d4T, AZT), Macrocytosis mild anemia and neutropenia (d4T, AZT), pancreatitis (d4T, 3TC, ddI), rare hypersensitivity (ABC), renotocixicity (TDF) rare red cell hyperplasia (3TC), hyperpigmentation (ABC):
	Emtricitabine (FTC) Lamivudine (3TC) Stavudine (d4T) Tenofovir (TDF)* Zidovudine (AZT) *Nucleotide		Propensity to cause hyperlactatemia in the decreasing order of magnitude: *dT4=ddI>>AZT>3TC=ABC=TDF=FTC
Non-nucleoside reverse transcriptase inhibitors (NNRTIs)	Nevirapine (NVP), Efavirenz (EFV) Etravirine (ETV) Rilpivirine (RVP)	Non-competitively inhibit RNA-dependent DNA Polymerase by allosteric Hindrance	Hypersensitivity presenting as skin rash which may be self-limiting or life threatening as in Steven Johnson Syndrome: *NVP > EFV=ETV=RVP Rash-associated hepatitis (NVP), neuropsychiatric side-effects (EFV), self-limiting gynaecomastia (EFV)
Protease inhibitor (PI)	Atazanavir (ATV) Darunavir (DRV) Fosamprenavir (fAPV) Indinavir (IDV) Nelfinavir (NFV) Ritonavir (RTV) Saquinavir (SQV) Tipranavir (TPV) Ritonavir (RTV)-booster only	Inhibits viral proteases that cleave viral polypeptides into functional proteins hence preventing formation of mature infectious viruses.	Lipohypertrophy, dyslipidemia (LPV/r*, DRV, IDV, SQV/r*), glucose intolerance, insulin resistance, hepatitis (LPV/r*), Nausea, vomiting and diarrhoea (LPV/r*, SQV/r*, DRV), skin rash (DRV), unconjugated hyperbilirubinemia (ATV, IDV), nephrolithiasis and diabetes (IDV). *Administered with RTV as a booster/pharmacokinetic enhancer
Entry inhibitor by blocking the host chemokine receptor-5	Maraviroc (MAC)	Inhibits the binding and entry of HIV	Limited experience with long term exposure but viral tropism to CCR-5 needs to be monitored/determined
Fusion inhibitor	Enfuvirtide (T-20)	Blocks the attachment, binding, and entry of the viral capsid into the host CD4+ cell.	Limited experience with long-term exposure but cutaneous injection site reactions, fatigue, insomnia, nausea, and diarrhea have been reported.
Integrase inhibitor	Raltegravir (RAL) Dolutegravir (DTG)	Block integration of proviral DNA into the CD4 cell genomic DNA by inhibiting DNA integrase	Headache, nausea and vomiting, rare Steven Johnson Syndrome, hepatitis and Rhabdomyolysis (RAL, DTG), raised serum creatinine (DTG).

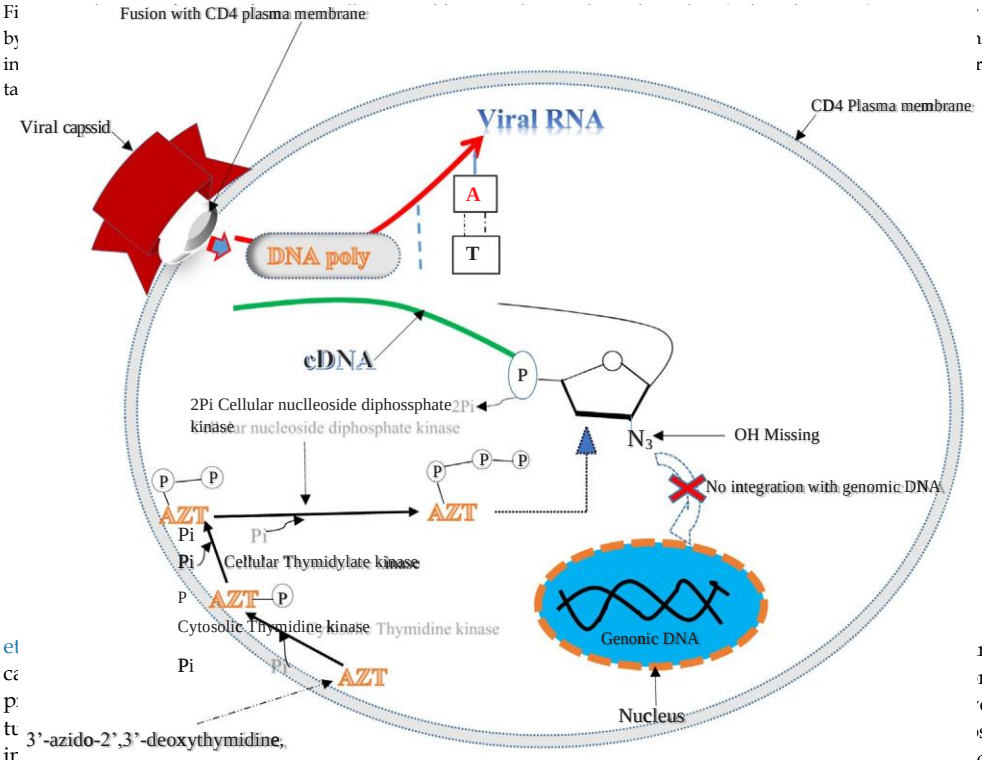
monophosphorylated to avoid the first rate-limiting phosphorylation step (Anderson et al., 2004). AZT, ddC, d4T, 3TC and TDF unlike FTC and ABC have unmodified bases to enhance base-pairing with the template strand and also avoid steric hindrances with the reverse transcriptase active site (El Safadi et al., 2007), (Table 1).

NRTIs therefore compete with the natural nucleotides for binding to the reverse transcriptase active site despite their relatively low intracellular concentrations [El Safadi et al. (2007)]. However, NRTIs not only inhibit genomic DNA replication, but also mitochondrial DNA (mtDNA) polymerase-γ, which is responsible for mtDNA replication and repair. mtDNA polymerase-γ which is responsible for the synthesis of critical enzymes that control mitochondrial oxidative phosphorylation and ATP production has higher affinity for NRTIs at therapeutic doses and is therefore more sensitive after chronic/acute exposure to these agents (Apostolova et al., 2011; Pe´rez-Matute et al., 2013). The affinity of r prokaryotic/eukaryotic DNA polymerases for NRTIs has been determined and ranked in the decreasing order of magnitude as HIV reverse transcriptase>>DNA polymerase-γ>DNA-polymerase-β > DNA polymerase-α = DNA polymerase-ε (Kakuda, 2000).

Non-Nucleoside Reverse Transcriptase Inhibitors (NNRTIs) inhibit reverse transcriptase by directly binding to the enzyme and causing allosteric hindrance (Marie-Pierre de Béthune, 2010). Protease Inhibitors (PIs) inhibit the HIV-1 enzyme proteases, which excise long chains of HIV proteins into smaller peptides hence preventing the assembling of new virus particles (Loonam and Mullen, 2012). Entry inhibitors bind to the host Chemokine Receptor type 5 (CCR5), therefore preventing interaction of viral gp120 and host CCR5 which are necessary for the entry into the host cell (Dorr and Perros, 2008). Fusion inhibitors prevent entry of viral nucleic acid into the host CD4 cell by disrupting the HIV-1 molecular machinery at the final stage of fusion with the target cell, preventing infection (Jacob et al., 2003). Integrase Inhibitors (IIs) block the integration of viral cDNA to the host genome by binding to the active site containing Mg^{2+} within HIV-1 integrase, thus blocking it from binding to target DNA strand (Pommier et al., 2005; Summa et al., 2008) (Table 1).

The current clinical treatment guidelines evolved from mono- therapy with AZT {which was originally developed in 1964 as an an- ticancer agent and later approved by FDA for HIV treatment in 1987 (Gardner et al., 2014)}. HIV treatment was later escalated, to Highly Active Antiretroviral Therapy (HAART) combination therapy (Rodríguez-Nóvoa et al., 2006), and now cART regimen, (which is formulated into a single fixed-dose combination pill) to enhance patient compliance and increase efficacy (Clinicians Society, 2013; Gandhi and Gandhi, 2014; Mitsuya et al., 1985). Even though the introduction of cARV has led to profound reduction in morbidity and mortality asso- ciated with HIV infections, chronic exposure to these agents has re- portedly been associated with increased risks of metabolic disturbances. The use of NRTIs for example, is known to cause mtDNA damage leading to mitochondrial myopathy, neuropathy, cardiomyopathy, life threatening hyperlactatemia/lactic acidosis, and lipodystrophy syn- drome (Duong et al., 2006; Estrada and Portilla, 2011; Hernández Pérez, Dawood, 2010; Mallal et al., 2000; Sagar et al., 2010; Saint-Marc

Fi … to into the CD4 cell, AZT is rapidly phosphrylated by … nto the growing viral cDNA strand. But since the in … ration of proviral cDNA with genomic DNA cannot ta …



et … nhibit mtDNA polymerase-γ leading to im-
ca … n and depletion. This causes diminished
p… e phosphorylation, reduced ATP production
tu … sis (Lewis and Dalakas, 1995; Lewis et al.,
ir … cerbated by the inefficiency of DNA poly-
re … removing the incorporated nucleotides (El

potheses and discusses citrus fruit-derived naringenin as a potential agent against NRTI-induced mitochondrial myopathy. Oxidative stress plays a critical role not only in HIV pathogenesis but also in the metabolic complications associated with NRTI therapy. Experimental evidence adduced in our laboratory suggests a potentially protective role for naringenin in this regard.

2. MtDNA polymerase-γ hypothesis

Mitochondrial toxicity commonly occurring in the liver, and skeletal muscles has been recognized as the most common metabolic complication associated with NRTIs (El Safadi et al., 2007). The efficiency of incorporation of NRTI triphosphates by mtDNA polymerase-γ leading to mtDNA depletion has been linked to mitochondrial toxicity (Johnson et al., 2001; Kakuda, 2000; Lee et al., 2003; Moyle, 2005). Not all NRTIs cause mitochondrial toxicity to the same extent depending on the cell types but they are generally ranked in the decreasing order of magnitude of ddC (discontinued) > ddI > d4T (withdrawn) > > AZT≥ FTC = ABC = TDF (Kakuda, 2000; Lim and Copeland, 2001; Mallon, 2007; Venoff et al., 2007;). Consequently, despite its superior viral load suppressing capacity, d4T has been withdrawn as a first-line therapy for HIV infections (Liu et al., 2017; World Health Organisation, 2010;). In vitro studies have previously shown that triphosphorylated forms of ddC, d4T and 3TC are better substrates of mtDNA polymerase-γ than AZT, TDF and ABC, while 3TC is a stronger substrate for mtDNA polymerase-γ exonuclease hence is more rapidly removed leading to lower mitochondrial toxicity (Birkus et al., 2002; Johnson et al., 2001; Lim and Copeland, 2001; Martin et al., 1994).

In the MtDNA polymerase-γ hypothesis, it was postulated that

Safadi et al., 2007; Eriksson et al., 1995;). Experimental and clinical evidence have supported this hypothesis even though it is not yet understood why some patients are more prone to NRTI mitochondrial toxicity than others. A number of host factors such as female sex, older age, hepatitis B or C co-infection, nadir CD4+T cell counts and diminished renal function have been identified as predisposing factors to mitochondrial toxicity in patients treated with NRTIs (Bonnet et al., 2003; Currier, 2007; Fleischer et al., 2004; Galli et al., 2003; Gervasoni et al., 1999; Lichtenstein et al., 2003). Hyperlactatemia for example has been reported more in women than men treated with d4T (Bolhaar and Karstaedt, 2007; John and Mallal, 2002).

NRTIs have been shown to cause mutations of mtDNA due to inhibition of critical enzymes of the electron transport chain and reduced fidelity of the DNA polymerase-γ exonuclease repair activity leading to mitochondrial myopathy (Martin et al., 2003). Mutations in the mtDNA have been reported to be over 10 times higher than that of the nuclear DNA (Wallace, 1992) making mitochondria more vulnerable to oxidative damage. Other than somatic mutations caused by NRTIs, there could also be genetic predisposition to NRTI toxicity but clinical evidence is missing. This could largely be due to the fact that such studies have mostly been done on Single Nucleotide Polymorphism (SNPS) without evidence from multivariate large scale data. A few cases of genetic predisposition to NRTIs that have clinically been defined include the polymorphism in the *HLA-B* gene and hypersensitivity reactions to abacavir, which nevertheless occurs in a small number of population groups (Hetherington et al., 2001). Polymorphism in the *Pol* γ coding sequence has been linked to the d4T-mediated mitochondrial toxicity (Bailey et al., 2009; Yamanaka et al., 2007). In this particular case, an HIV positive patient treated with d4T presented with lactic

acidosis and was found to harbor a homozygous R964C mutation in the *Pol* γ gene (Bailey et al., 2009; Yamanaka et al., 2007;). Furthermore, E1143G *Poly* γ gene polymorphism has been linked to d4T-associated lipodystrophy (Chiappinin et al., 2009) but the underlying molecular mechanisms have not yet been determined. A mutation in the nuclear gene encoding mtDNA polymerase-γ has been described in a patient treated with d4T suggesting that pre-existing nuclear gene mutation can be a predisposing factor in NRTI-associated mitochondrial toxicity (Yamanaka et al., 2007). Age-related mtDNA mutations could also be predisposing factors to NRTI-associated mitochondrial toxicity considering long cumulative exposure (Chan, 2006; Hugan et al., 2005). Clearly, there appear to be as yet unknown complex pathophysiological processes relative to pharmacogenetics of NRTI-induced mitochondrial toxicity other than mtDNA polymerase-γ hypothesis.

2.1. Beyond the mtDNA polymerase-γ hypothesis

The realization that the affinity of mtDNA polymerase-γ and their propensity to inhibition by NRTIs does not linearly correlate with mitochondrial clinical toxicity led to the conclusion that there could be other mechanisms by which NRTIs cause mitochondrial toxicity. For example, of all the NRTIs, AZT is not the most potent inhibitor of mtDNA polymerase-γ yet it causes the most adverse mitochondrial toxicity (Cihlar and Ray, 2010). AZT is less efficiently incorporated into mtDNA but has been suggested to be an inhibitor of mtDNA polymerase-γ exonuclease activity (Lim and Copeland, 2001). Other in vitro studies have suggested that AZT suppresses complex IV of the mitochondrial oxidative phosphorylation chain without mtDNA depletion or impaired ATP synthesis (Barile et al., 1997, 1998; Birkus et al., 2002; Pan-Zhou et al., 2000). Collectively, these observations do suggest that AZT may additionally be causing electron leakage into the matrix and decreasing mitochondrial oxidative phosphorylation due to increased reactive oxygen species generation and oxidative damage (Wallace, 1999; Yamaguchi et al., 2002). It has also been hypothesized that inhibition of mitochondrial telomerase by NRTIs is associated with mitochondrial toxicity and premature aging in HIV patients treated with NRTIs (Bollman, 2013).

That not every case of mtDNA depletion or reduced expression of oxidative phosphorylation enzymes results from altered mitochondrial gene expression or impaired oxidation phosphorylation capacity, suggests that NRTIs could be affecting mitochondrial function by other means (Mallon et al., 2005; Stankov et al., 2010).

2.2. The role of oxidative stress in NRTI-associated metabolic complications

mtDNA polymerase-γ is nuclear encoded and is the only polymerase found in the mitochondria hence its inhibition amongst others causes enhanced production of ROS (Brinkman et al., 1998; Lewis and Dalakas, 1995). Mitochondria are the main cellular source and targets of reactive oxygen species (Apostolova et al., 2011; Stankov et al., 2008). NRTI-associated mitochondrial toxicity is hardly reversible and is often life threatening. But beyond the mtDNA polymerase-γ hypothesis, oxidative stress appears to play a significant role besides other suggested mechanisms such as mtRNA polymerase depletion and direct inhibitory effects on mitochondrial proteins controlling oxidative phosphorylation (Galluzzi et al., 2005; Pe´rez-Matute et al., 2013). AZT for example has been shown in vitro to directly inhibit mitochondrial adenylate cyclase and adenosine nucleotide translocator in the mitochondria, promote oxidative stress and impair mitochondrial oxidative phosphorylation (Côté, 2005; Feeney and Mallon, 2010; Pe´rez-Matute et al., 2013). NRTIs have been reported to inhibit mitochondrial oxidative phos- phorylation complexes I and IV and AZT has been shown in vivo to induce mitochondrial oxidative stress and cause cardiomyopathy (Côté, 2005; Lund and Wallace, 2008; Maagaard and Kvale, 2009; Pe´rez- Matute. et al., 2013;). mtDNA polymerase-γ is known to be sensitive to oxidative stress which alters its amino acid sequence leading to decreased polymerase activity and DNA binding affinity (Blas-Garcia et al., 2011; Day and Lewis, 2004; Graziewicz et al., 2002). cART has been linked to reactive oxygen species-associated increased incidence of cardiovascular diseases, central nervous system disturbances, inflammation, metabolic and lipodystrophy syndromes (Kline et al., 2009; Manda et al., 2011; Trifunovic and Larsson, 2008). ROS therefore is central to cARV-associated metabolic complications and any attempts to neutralize their production could potentially mitigate the develop- ment of these pathological manifestations. Oxidative stress is defined as an imbalance between reactive oxygen species elements (super oxide, hydrogen peroxide, lipid peroxides hydroxyl radicals and peroxynitrite) and antioxidant defense systems. Limiting its effects is therefore an attractive option in reducing cARV-induced mitochondrial toxicity. Oxidative stress can impair mtDNA replication by oxidizing mtDNA templates, reducing oxidative phosphorylation by causing electron leakage and reduced ATP production, ultimately leading to mitochondrial myopathy (Kohler and Lewis, 2007). Mitochondria are known to be protected from oxidative damage by antioxidant enzymes such as manganese superoxide dismutase (MnSOD), and glutathione peroxidase (Kohler and Lewis, 2007) but these may be damaged or depleted during increased reactive oxygen species production.

Compliant HIV-1 infected patients that are maintained on intensive cARV therapy are deemed to have profoundly suppressed viral activity hence increased oxidative stress that causes mitochondrial toxicity is largely due to cARV therapy as opposed to viral pathogenesis (Mandas et al., 2009). Would reducing reactive oxygen species production or boosting antioxidant capacity in these patients reduce mitochondrial toxicity? An in vitro study by Smith et al. (2017) has suggested that N-acetylcysteine mitigates NRTI-induced oxidative stress in *C. elegans* following previously failed attempts to use antioxidants such as vitamin E, beta-carotene, N-acetylcysteine and nutritional supplements with mitochondrial co-factors like carnitine, riboflavine and coenzyme Q to reduce hyperlactatemia in HIV patients treated with cARVs (Lopez et al., 2003). Changes in antioxidant profile in HIV positive patients on cARV therapy have previously been described (Mandas et al., 2009; Sundaram et al., 2008) and it has been suggested that therapeutic interventions to maintain oxidative balance in HIV positive patients on cARV therapy could mitigate the development of mitochondrial toxicity (Aukrust et al., 2003; Pe´rez-Matute et al., 2013). It is therefore becoming apparent that natural compounds with antioxidant capacities could be beneficial not only in preventing oxidative damage caused by HIV infection itself, but also, in cARV-associated oxidative stress. A previous study by Chandra et al. (2009) has shown that thymoquinone, an active ingredient of black seed oil protected pancreatic β-cells against nelfinavir-induced oxidative damage. Protective effects of fla- vonoid resveratrol against PI-induced sarcoplasmic/endoplasmic re- ticulum stress in human myoblasts have been described (Touzet and Philips, 2010). Our own laboratory has recently reported that naringin, a citrus fruit-derived flavonoid abrogates HIV-1 protease inhibitors-in- duced atherogenic dyslipidemia and oxidative stress in vivo (Nzuza and Owira, 2019). We have also shown that naringin prevents HIV-1 pro- tease inhibitors-induced metabolic complications in vivo (Nzuza et al., 2017). Our in vitro studies have suggested that naringin protects against HIV-1 protease inhibitors-induced pancreatic β-cell dysfunction and apoptosis (Nzuza et al., 2016). Previously, we reported that nar- ingin ameliorates HIV-1 NRTI-induced mitochondrial toxicity (Adebiyi et al., 2016), improves AZT- and d4T-induced skeletal muscle compli- cations in rats (Adebiyi et al., 2017). Prior to that, we showed that naringinreversed hepatocyte apoptosis and oxidative stress associated with HIV-1 NRTI-induced metabolic complications (Adebiyi et al., 2015). We are hereby convinced that naringin unlike vitamins E or C which previously failed as dietary supplements against oxidative stress caused by cART, is a stronger antioxidant owing to the fact that it cannot only directly quench free radicals but also boost antioxidant capacity in conditions of metabolic stress. In this review, we argue a case for naringenin, which is a naringin aglycone that has been reported

to be having more potent antioxidant properties owing to the lack of the glucose molecule that causes steric hindrance (Adil et al., 2016; Jung et al., 2003).

3. Putative protective antioxidant effects of naringenin

Naringenin (4′,5, 7-trihydroxy flavone) found mostly in citrus fruits, is very lipophilic and is readily absorbed through intestinal epithelium by passive diffusion (Nait et al., 2009). It enters general circulation through multidrug resistance associated proteins (Mrp1) or can be ex- truded from the enterocytes by P-glycoprotein and Mrp2 back into the lumen (Nait et al., 2009). Naringenin is metabolized in the intestines, colonic epithelium and also by phase II hepatic metabolism by con- jugation to UDP-glucuronyl transferase (UGT), sulfotransferase, and catechol-O-methyl transferase (Bredsdorff et al., 2010; Scalbert et al., 2002; Simons et al., 2010). Naringenin does not therefore affect phase I hepatic metabolism of xenobiotics. Naringenin distributes well in body tissues and just like its metabolites, binds avidly to plasma albumin (Bolli et al., 2010; El Mohsen et al., 2004; Khan et al., 2011; Simons et al., 2010; Zou et al., 2012;).

Naringenin owes its potent antioxidant effects to its OH groups, which have high affinity for reactive oxygen species and reactive ni- trogen species (Heim et al., 2002; Rice-Evans et al., 1996), (Fig. 2). Naringenin therefore has been demonstrated in vivo and in vitro to possess antioxidant effects by directly scavenging free radicals hence preventing oxidative stress (Chen et al., 2014). Besides its demonstrated antioxidant potential, naringenin has also been shown to block lipid peroxidation in multiple organs (Al-Dosari et al., 2017; Ali et al., 2017; Al-Roujayee, 2017; Miler et al., 2016; Pari et al., 2006; Ramprasath et al., 2014;) by reducing plasma membrane fluidity (Kaneko et al., 1994).

Naringenin reduces oxidative stress not only by directly quenching free radicals but also by boosting antioxidant system following upre- gulation of the cellular expression of antioxidant enzymes such as su- peroxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx) and glutathione transferase (GST) (Al-Roujayee, 2017; Miler et al., 2016; Nzuza et al., 2016; Pari and Gnanasoundari, 2006). We have observed in vitro that in hyperglycemia-induced oxidative stress, glutathione (GSH) levels initially increases in the presence of PIs then decrease and steadily decline in controls but in the presence of nar- ingin, this decline is opposed despite increased oxidative stress (Nzuza et al., 2016). This suggests in our view that GSH is inducible and that naringin by either directly quenching reactive oxygen species relieves the oxidative burden and allows the cells to express GSH similarly to other findings (Al-Dosari et al., 2017; Ali et al., 2017). GSH is a tri- peptide (L-γ-glutamyl-L-cysteinyl-glycine) that acts as an antioxidant, detoxifies oxygen-derived ROS, facilitates thiol disulfide exchange and storage/transfer of cysteine (Hernández-Aquino and Muriel, 2018). GSH can directly scavenge ROS or function as a co-substrate of GPx (in H_2O_2 reduction to molecular oxygen and water) and of GST in the biotransformation of xenobiotics then it gets oxidized to GSSG, hence

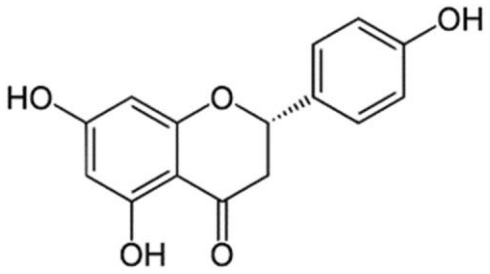


Fig. 2. Chemical structure of naringenin showing the 3 OH groups that have high affinity for ROS and RNS species to which they donate electrons. They also chelate positively charged compounds.

redox balance is maintained by GSH/GSSG ratio (Kretzschmar, 1996; Yuan and Kaplowitz, 2009). SOD catalyses conversion of molecular oxygen to H_2O_2, which is then neutralized by CAT hydrolysis to water and oxygen (Davies, 2000).

3.1. Molecular Mechanisms of naringenin antioxidant effects

The precise mechanisms involved in naringenin antioxidant effects are still elusive. It has been suggested that naringenin influences cel- lular expression of microRNAs (miRNA) and nuclear factor-erythroid 2 related factor 2 (Nrf2) (Hernández-Aquino and Muriel, 2018). Nar- ingenin has been reported to inhibit the expression of miR-17-3p genes which suppress the expression of antioxidant enzymes SOD and GPx (Curti et al., 2017; Xu et al., 2010;).

Furthermore, naringenin has been shown to increase Nrf2 protein expression by promoting its translocation to the nucleus after phos- phorylation by Mitogen Activated Protein Kinases (MAPK) (Chen et al., 2015; Cullinan et al., 2003; Dong et al., 2015; Lee et al., 2012; Liu et al., 2011; Lou et al., 2014; Podder et al., 2014; Ramprasath et al., 2014;). It may also facilitate Nrf2 conformational changes that dissociate it from Kelch-like ECH associating protein 1 (keap1) in response to increased oxidative stress (Hernández-Aquino and Muriel, 2018). Activated Nrf2 then binds to antioxidant response element sequences in the nucleus to induce transcription of antioxidant enzymes such as GST, SOD and CAT and detoxification proteins (Ooi et al., 2017; Tang et al., 2014; Tebay et al., 2015;).

Even though naringenin many not be the most potent natural an- tioxidant compared to other flavonoids such as quercetin (Hernández-Aquino and Muriel, 2018) its ability to regulate transcription factors with antioxidant effects makes it superior as a first choice in mitigating oxidative stress that causes metabolic disturbances. There is no known toxicity of naringenin to both humans and experimental laboratory animals.

4. Clinical perspectives

This review has presented compelling evidence why NRTI-mi- tochondrial toxicity is not just a phenomenon of mtDNA-γ polymerase hypothesis, but a consequence of complex interplay between host ge- netic factors and oxidative stress. We have presented evidence why naringenin should strongly be considered as either a dietary supplement or adjunct therapy for the management of metabolic complications associated with NRTIs. In this respect, naringenin could be formulated as a separate adds-on pill or compounded with the single pill in cARV to ameliorate these deleterious side-effects. Even though the newly de- veloped drugs that are incorporated as components of cARV are largely devoid of these metabolic complications, their long-term side-effects are not known as most of them seem to become apparent only during post licensing exposure and not during drug development. With new revelations that naringenin could actually possess antiviral properties (Goldwasser et al., 2011; Khachatoorian et al., 2012), this approach could potentially benefit HIV positive patients with HCV/HBV co-in- fection. Large-scale clinical trials to determine, efficacy and dosage formulations of naringenin are suggested.

CRediT authorship contribution statement

Mathabo Ruth Lutu: Writing - original draft. Sanelisiwe Nzuza: Data curation. Pascale Edith Mofo Mato: Data curation. Khmera Govender: Data curation. Londiwe Mphumelelo Gumede: Data curation. Sinenhlanhla Innocentia Kumalo: Data curation. Nontokozo Nomfundo Mlambo: Data curation. Ranjendraparsad Hurchund: Data curation. Peter Mark Oroma Owira: Conceptualization, Project administration, Writing - review & editing.

Acknowledgements

We are indebted to the University of KwaZulu-Natal Postgraduate office for the scholarship funding.

Conflict of interests

None.

Author agreement

MRL = Drafted the original manuscript
SN, PEMM, KG, LMG, SIK, NNM, RH = Data curation, literature search and review
PMOO = Conceptualization, administration, Writing -review & editing

References

Adebiyi, O.O., Adebiyi, O.A., Owira, P., 2016. Naringin ameliorates HIV-1 nucleoside reverse transcriptase inhibitors-induced mitochondrial toxicity. Curr. HIV Res. 4 (6), 506–516.
Adebiyi, O.O., Adebiyi, O.A., Owira, P., 2017. Naringin improves zidovudine- and stavudine-induced skeletal muscle complications in rats. Hum. Exp. Toxicol. 36 (1), 93–105.
Adebiyi, O.A., Adebiyi, O.O., Owira, P.M.O., 2015. Naringin reverses hepatocyte apoptosis and oxidative stress associated with HIV-1 Nucleotide Reverse Transcriptase Inhibitors-induced metabolic complications. Nutrients 7, 10352–10368.
Adil, M., et al., 2016. Ameliorative effect of naringin in acetaminophen induced hepatic and renal toxicity in laboratory rats: role of FXR and KIM-1. Ren. Fail. 38 (6), 1007–1020.
Aid for Aids, 2016. Treatment guideline, 11th Edition.
Al-Dosari, et al., 2017. Flavonoid naringenin attenuates oxidative stress, apoptosis and improves neurotrophic effects in the diabetic rat retina. Nutrients 9, 1161. https://doi.org/10.3390/nu9101161.
Ali, et al., 2017. Amelioration of benzo[a]pyrene-induced oxidative stress and pulmonary toxicity by naringenin in Wistar rats: a plausible role of COX-2 and NF-κB. Hum. Exp. Toxicol. 36 (4), 349–364.
Al-Roujayee, A.S., 2017. Naringenin improves the healing process of thermally-induced skin damage in rats. J. Int. Med. Res. 45 (2), 570–582.
Anderson, P.L., Kakuda, T.N., Lichtenstein, K.A., 2004. The cellular pharmacology of nucleoside- and nucleotide-analogue reverse-transcriptase inhibitors and its re- lationship to clinical toxicities. Clin. Infect. Dis. 38 (5), 743–753.
Anuurad, E., Bremer, A., Berglund, L., 2010. HIV protease inhibitors and obesity. Curr. Opin. Endocrinol. Diabetes Obes. 17, 478–485.
Apostolova, N., Blas-Garcia, A., Esplugues, J.V., 2011. Mitochondrial interference by anti-HIV drugs: mechanisms beyond Pol-gamma inhibition. Trends Pharmacol. Sci. 32, 715–725.
Aukrust, et al., 2003. Disturbed glutathione metabolism and decreased antioxidant levels in human immunodeficiency virus-infected patients during highly active anti- retroviral therapy-potential immunomodulatory effects of antioxidants. J. Infect. Dis. 188, 232–238.
Bailey, et al., 2009. R964C mutation of DNA polymerase gamma imparts increased stavudine toxicity by decreasing nucleoside analog discrimination and impairing poly- merase activity. Antimicrob. Agents Chemother. 53 (6), 2610–2612.
Barbaro, G., 2006. Metabolic and cardiovascular complications of highly active antiretroviral therapy for HIV infection. Curr. HIV Res. 4, 79–85.
Barile, et al., 1997. 3'-Azido-3'-deoxythmidine uptake into isolated rat liver mitochondria and impairment of ADP/ATP translocator. Biochem. Pharmacol. 53 (7), 913–920.
Barile, et al., 1998. Mitochondria as cell targets of AZT (zidovudine). Gen. Pharmacol. 31 (4), 531–538.
Bekker, et al., 2014. Provision of antiretroviral therapy in South Africa: the nuts and bolts. Antivir. Ther. 19 (Suppl 3), 105–116.
Birkus, G., Hitchcock, M.J., Cihlar, T., 2002. Assessment of mitochondrial toxicity in human cells treated with tenofovir: comparison with other nucleoside reverse transcriptase inhibitors. Antimicrob. Agents Chemother. 46 (3), 716–723.
Blas-Garcia, A., Apostolova, N., Esplugues, J.V., 2011. Oxidative stress and mitochondrial impairment after treatment with anti-HIV drugs: clinical implications. Curr. Pharm. Des. 17 (36), 4076–4086.
Bolhaar, M.G., Karstaedt, A.S., 2007. A high incidence of lactic acidosis and symptomatic hyperlactatemia in women receiving highly active antiretroviral therapy in Soweto, South Africa. Clin. Infect. Dis. 45 (2), 254–260.
Bolli, et al., 2010. Flavonoid binding to human serum albumin. Biochem. Biophys. Res. Commun. 398 (3), 444–449.
Bollman, F.M., 2013. Telomerase inhibition may contribute to accelerated mitochondrial aging induced by anti-retroviral HIV treatment. Med. Hypotheses 81 (2), 285–287.
Bonnet, et al., 2003. Risk factors for lactic acidosis in HIV-infected patients treated with nucleoside reverse transcriptase inhibitors: a case control study. Clin. Infect. Dis. 36, 1324–1328.
Bredsdorff, et al., 2010. Absorption, conjugation and excretion of the flavanones, naringenin and hesperetin from alpha-rhamnosidase-treated orange juice in human subjects. Br. J. Nutr. 103 (11), 1602–1609.
Brinkman, et al., 1998. Adverse effects of reverse transcriptase inhibitors: mitochondrial toxicity as common pathway. AIDS 12 (14), 1735–1744.
Chan, D.C., 2006. Mitochondria: dynamic organelles in disease, aging, and development. Cell 125 (7), 1241–1252.
Chandra, S., Mondal, D., Agrawal, K.C., 2009. HIV-1 protease inhibitor induced oxidative stress suppresses glucose-stimulated insulin release: protection with thymoquinone. Exp. Biol. Med. 234, 442–453.
Chen, et al., 2014. Naringin inhibits ROS-activated MAPK pathway in high glucose-induced injuries in H9c2 cardiac cells. Basic Clin. Pharmacol. Toxicol. 114 (4), 293–304.
Chen, et al., 2015. Naringin protects against anoxia/reoxygenation-induced apoptosis in H9c2 cells via the Nrf2 signaling pathway. Food Funct. 6 (4), 1331–1344.
Chiappinin, et al., 2009. Relationship between polymerase gamma (POLG) polymorphisms and antiretroviral therapy-induced lipodystrophy in HIV-1 infected patients: a case-control study. Curr. HIV Res. 7 (2), 244–253.
Cihlar, T., Ray, A.S., 2010. Nucleoside and nucleotide HIV reverse transcriptase inhibitors: 25 years after zidovudine. Antivir. Res. 85 (1), 39–58.
Clinicians Society, S.H., 2013. Fixed-dose combination for adults accessing antiretroviral therapy. South. Afr. J. HIV Med. ⟨sajhivmed.org.za⟩.
Côté, H.C., 2005. Possible ways nucleoside analogues can affect mitochondrial DNA content and gene expression during HIV therapy. Antivir. Ther. 10 (Suppl 2), M3–M11.
Cullinan, et al., 2003. Nrf2 is a direct PERK substrate and effector of PERK-dependent cell survival. Mol. Cell Biol. 23 (20), 7198–7209.
Currier, J.S., 2007. Sex differences in antiretroviral therapy toxicity: lactic acidosis, sta- vudine and women. Clin. Infect. Dis. 45, 261–262.
Curti, et al., 2017. Enantioselective modulatory effects of naringenin enantiomers on the expression levels of miR-17-3p involved in endogenous antioxidant defences. Nutrients 9 (pii: E215).
Davies, K.J., 2000. Oxidative stress, antioxidant defences, and damage removal, repair, and replacement systems. IUBMB Life 50 (4–5), 279–289.
Day, B.J., Lewis, W., 2004. Oxidative stress in NRTI-induced toxicity: evidence from clinical experience and experiments in vitro and in vivo. Cardiovasc. Toxicol. 4 (3), 207–216.
Dong, et al., 2015. Naringin prevents carbon tetrachloride-induced acute liver injury in mice. J. Funct. Foods 12, 179–191.
Dorr, P., Perros, M., 2008. CCR5 inhibitors in HIV-1 therapy. Expert Opin. Drug Discov. 3, 1345–1361.
Duong, et al., 2006. Alteration of cytochrome oxidase subunit I labeling is associated with severe mitochondriopathy in NRTI-related hepatotoxicity in HIV patients. Mod. Pathol. 19, 1277–1288.
El Mohsen, et al., 2004. The differential tissue distribution of the citrus flavanone nar- ingenin following gastric instillation. Free Radic. Res. 38 (12), 1329–1340.
El Safadi, Y., Vivet-Boudou, V., Marquet, R., 2007. HIV-1 reverse transcriptase inhibitors. Appl. Microbiol. Biotechnol. 75 (4), 723–737.
Eriksson, S., Xu, B., Clayton, D.A., 1995. Efficient incorporation of anti-HIV deoxynucleotides by recombinant yeast mitochondrial DNA polymerase. J. Biol. Chem. 270, 18929–18934.
Estrada, V., Portilla, J., 2011. Dyslipidemia related to antiretroviral therapy. AIDS Rev. 13, 49–56.
Feeney, E.R., Mallon, P.W., 2010. Impact of mitochondrial toxicity of HIV-1 antiretroviral drugs on lipodystrophy and metabolic dysregulation. Curr. Pharm. Des. 16 (30), 3339–3351.
Fleischer, R., Boxwell, D., Sherman, K.E., 2004. Nucleoside analogs and mitochondrial toxicity. Clin. Infect. Dis. 38 (8), e79–e80.
Gardner, et al., 2014. HIV treatment and associated mitochondrial pathology: review of 25 years of in vitro, animal, and human studies. Toxicol. Pathol. 42 (5), 811–822.
Galli, et al., 2003. Gender differences in antiretroviral drug-related adipose tissue alterations. Women are at higher risk than men and develop particular lipodystrophy patterns. J. Acquir Immune Defic. Syndr. 34 (1), 58–61.
Galluzzi, et al., 2005. Changes in mitochondrial RNA production in cells treated with nucleoside analogues. Antivir. Ther. 10 (1), 191–195.
Gandhi, M., Gandhi, R.T., 2014. Single-pill combination regimens for treatment of HIV-1 infection. New Engl. J. Med. 371, 248–259.
Gervasoni, et al., 1999. Redistribution of body fat in HIV-infected women undergoing combined antiretroviral therapy. AIDS 13 (4), 465–471.
Goldwasser, et al., 2011. Naringenin inhibits the assembly and long-term production of infectious hepatitis C virus particles through a PPAR-mediated mechanism. J. Hepatol. 55 (5), 963–971.

Graziewicz, M.A., Day, B.J., Copeland, W.C., 2002. The mitochondrial DNA polymerase as a target of oxidative damage. Nucleic Acids Res. 30 (13), 2817–2824.
Heim, K.E., Tagliaferro, A.R., Bobilya, D.J., 2002. Flavonoid antioxidants: chemistry, metabolism and structure-activity relationships. J. Nutr. Biochem. 13 (10), 572–584.
Hernández-Aquino, E., Muriel, P., 2018. Beneficial effects of naringenin in liver diseases: molecular mechanisms. World J. Gastroenterol. 24 (16), 1679–1707.
Hernández Pérez, E., Dawood, H., 2010. Stavudine-induced hyperlactatemia/lactic acidosis at a tertiary communicable diseases clinic in South Africa. J. Int. Assoc. Physicians AIDS Care 9 (2), 109–112.
Hetherington, et al., 2001. Hypersensitivity reactions during therapy with the nucleoside reverse transcriptase inhibitor abacavir. Clin. Ther. 23, 1603–1614.
Hulgan, et al., 2005. Mitochondrial haplogroups and peripheral neuropathy during antiretroviral therapy: an adult AIDS clinical trials group study. AIDS 19 (13), 1341–1349.
Jacob, et al., 2003. Enfuvirtide, an HIV-1 fusion inhibitor, for drug-resistant HIV infection in North and South America. N. Engl. J. Med. 348, 2175–2185.
John, M., Mallal, S., 2002. Hyperlactatemia syndromes in people with HIV infection. Curr. Opin. Infect. Dis. 15, 23–29.
Johnson, et al., 2001. Toxicity of antiviral nucleoside analogs and the human mitochondrial DNA polymerase. J. Biol. Chem. 276 (44), 40847–40857.
Jung, U.J., et al., 2003. Naringin supplementation lowers plasma lipids and enhances erythrocyte antioxidant enzyme activities in hypercholesterolemic subjects. Clin. Nutr. 22, 561–568.
Khachatoorian, et al., 2012. Divergent antiviral effects of bioflavonoids on the hepatitis C virus life cycle. Virology 433 (2), 346–355.
Kakuda, T.N., 2000. Pharmacology of nucleoside and nucleotide reverse transcriptase inhibitor-induced mitochondrial toxicity. Clin. Ther. 22 (6), 685–708.
Kaneko, T., Kaji, K., Matsuo, M., 1994. Protection of linoleic acid hydroperoxide-induced cytotoxicity by phenolic antioxidants. Free Radic. Biol. Med. 16 (3), 405–409.
Khan, et al., 2011. Binding of citrus flavanones and their glucuronides and chalcones to human serum albumin. Food Funct. 2 (10), 617–626.
Kline, et al., 2009. Long-term exposure to AZT, but not d4T, increases endothelial cell oxidative stress and mitochondrial dysfunction. Cardiovasc. Toxicol. 9 (1), 1–12.
Kohler, J.J., Lewis, W., 2007. A brief overview of mechanisms of mitochondrial toxicity from NRTIs. Environ. Mol. Mutagen. 48 (3–4), 166–172.
Kretzschmar, M., 1996. Regulation of hepatic glutathione metabolism and its role in hepatotoxicity. Exp. Toxicol. Pathol. 48 (5), 439–446.
Lee, H., Hanes, J., Johnson, K.A., 2003. Toxicity of nucleoside analogues used to treat AIDS and the selectivity of the mitochondrial DNA polymerase. Biochemistry 42 (50), 14711–14719.
Lee, et al., 2012. Induction of heme oxygenase-1 inhibits cell death in crotonaldehyde-stimulated HepG2 cells via the PKC-δ-p38-Nrf2 pathway. PLoS One 7 (7), e41676.
Lewis, W., Dalakas, M.C., 1995. Mitochondrial toxicity of antiviral drugs. Nat. Med. 1 (5), 417–422.
Lewis, W., Day, B.J., Copeland, W.C., 2003. Mitochondrial toxicity of NRTI antiviral drugs: an integrated cellular perspective. Nat. Rev. Drug Discov. 2 (10), 812–822.
Lichtenstein, et al., 2003. Incidence of and risk factors for lipoatrophy (abnormal fat loss) in ambulatory HIV-1-infected patients. J. Acquir Immune Defic. Syndr. 32 (1), 48–56.
Lim, S.E., Copeland, W.C., 2001. Differential incorporation and removal of antiviral deoxynucleotides by human DNA polymerase gamma. J. Biol. Chem. 276 (26), 23616–23623.
Liu, et al., 2011. Activation of AMPK stimulates heme oxygenase-1 gene expression and human endothelial cell survival. Am. J. Physiol. Heart Circ. Physiol. 300 (1), H84–H93.
Liu, et al., 2017. The Δ133p53 isoform reduces Wtp53-induced stimulation of DNA Pol γ activity in the presence and absence of D4T. Aging Dis. 8 (2), 228–239.
Loonam, C.R., Mullen, A., 2012. Nutrition and the HIV-associated lipodystrophy syndrome. Nutr. Res Rev. 25, 267–287.
Lopez, et al., 2003. Could antioxidant supplementation reduce antiretroviral therapy-induced chronic stable hyperlactatemia? Biomed. Pharmcother. 57 (3–4), 113–116.
Lou, et al., 2014. Naringenin protects against 6-OHDA-induced neurotoxicity via activation of the Nrf2/ARE signaling pathway. Neuropharmacol 79, 380–388.
Lund, K.C., Wallace, K.B., 2008. Adenosine 3',5'-cyclic monophosphate (cAMP)-depen- dent phosphoregulation of mitochondrial complex I is inhibited by nucleoside reverse transcriptase inhibitors. Toxicol. Appl. Pharmacol. 226 (1), 94–106.
Maagaard, A., Kvale, D., 2009. Mitochondrial toxicity in HIV-infected patients both off and on antiretroviral treatment: a continuum or distinct underlying mechanisms? J. Antimicrob. Chemother. 64 (5), 901–909.
Maartens, G., Celum, C., Lewin, S.R., 2014. HIV infection: epidemiology, pathogenesis, treatment, and prevention. Lancet 384, 258–271.
Mallal, et al., 2000. Contribution of nucleoside analogue reverse transcriptase inhibitors to subcutaneous fat wasting in patients with HIV infection. AIDS 14 (10), 1309–1316.
Mallon, P.W.G., 2007. Antiretroviral therapy-induced lipid alterations: in-vitro, animal and human studies. Curr. Opin. HIV Aids 2 (4), 282–292.
Mallon, et al., 2005. In vivo, nucleoside reverse-transcriptase inhibitors alter expression of both mitochondrial and lipid metabolism genes in the absence of depletion of mitochondrial DNA. J. Infect. Dis. 191 (10), 1686–1696.
Manda, et al., 2011. Highly active antiretroviral therapy drug combination induces oxidative stress and mitochondrial dysfunction in immortalized human blood-brain barrier endothelial cells. Free Radic. Biol. Med. 50 (7), 801–810.
Mandas, et al., 2009. Oxidative imbalance in HIV-1 infected patients treated with antiretroviral therapy. J. Biomed. Biotech (ID 749575. Doi:1155/209/749575).
Marie-Pierre de Béthune, 2010. Non-nucleoside reverse transcriptase inhibitors (NNRTIs), their discovery, development, and use in the treatment of HIV-1 infection: a review of the last 20 years (1989–2009). Antivir. Res. 85, 75–90.
Martin, et al., 1994. Effects of antiviral nucleoside analogs on human DNA polymerases and mitochondrial DNA synthesis. Antimicrob. Agents Chemother. 38 (12), 2743–2749.
Martin, et al., 2003. Accumulation of mitochondrial DNA mutations in human immunodeficiency virus-infected patients treated with nucleoside-analogue reverse-transcriptase inhibitors. Am. J. Hum. Genet. 72 (3), 549–560.
Miler, et al., 2016. Citrus flavanones naringenin and hesperetin improve antioxidant status and membrane lipid compositions in the liver of old-aged Wistar rats. Exp. Gerontol. 84, 49–60.
Mitsuya, et al., 1985. 3'-Azido-3'-deoxythymidine (BW A509U): an antiviral agent that inhibits the infectivity and cytopathic effect of human T-lymphotropic virus type III/lymphadenopathy-associated virus in vitro. Proc. Natl. Acad. Sci. USA 82 (20), 7096–7100.
Moyle, G., 2005. Mechanisms of HIV and nucleoside reverse transcriptase inhibitor injury to mitochondria. Antivir. Ther. 10 (Suppl 2), M47–M52.
Nait, et al., 2009. Quercetin and naringenin transport across human intestinal Caco-2 cells. J. Pharm. Pharmacol. 61 (11), 1473–1483.
Nzuza, S., Owira, P., 2019. Naringin abrogates HIV-1 protease inhibitors-induced atherogenic dyslipidemia and oxidative stress in vivo. J. Funct. Foods 52, 156–164.
Nzuza, S., Zondi, S., Owira, P.M.O., 2017. Naringin prevents HIV-1 protease inhibitors-induced metabolic complications in vivo. PLoS One 12 (11), e0183355.
Nzuza, S., Ndwandwe, D.E., Owira, P.M., 2016. Naringin protects against HIV-1 protease inhibitors-induced pancreatic β-cell dysfunction and apoptosis. Mol. Cell Endocrinol. 437, 1–10.
Ooi, B.K., Goh, B.H., Yap, W.H., 2017. Oxidative stress in cardiovascular diseases: involvement of Nrf2 antioxidant redox signaling in macrophage foam cells formation. Int. J. Mol. Sci. 18 (11) (pii: E2336).
Pan-Zhou, et al., 2000. Differential effects of antiretroviral nucleoside analogs on mitochondrial function in HepG2 cells. Antimicrob. Agents Chemother. 44 (3), 496–503.
Pari, L., Gnanasoundari, M., 2006. Influence of naringenin on oxytetracycline mediated oxidative damage in rat liver. Basic Clin. Pharmacol. Toxicol. 98 (5), 456–461.
Pe´rez-Matute, et al., 2013. Role of mitochondria in HIV infection and associated meta- bolic disorders: focus on nonalcoholic fatty liver disease and lipodystrophy syn- drome. Oxidative Medicine and Cellular longevity. https://doi.org/10.1155/2013/ 493413.
Podder, B., Song, H.Y., Kim, Y.S., 2014. Naringenin exerts cytoprotective effect against paraquat-induced toxicity in human bronchial epithelial BEAS-2B cells through NRF2 activation. J. Microbiol. Biotechnol. 24 (5), 605–613.
Pommier, Y., Allison, A., Johnson, A.A., Marchand, C., 2005. Integrase inhibitors to treat HIV/Aids. Nat. Rev. Drug Disc. 4, 236–248.
Ramprasath, et al., 2014. Naringenin confers protection against oxidative stress through upregulation of Nrf2 target genes in cardiomyoblast cells. J. Physiol. Biochem. 70 (2), 407–415.
Rice-Evans, C.A., Miller, N.J., Paganga, G., 1996. Structure-antioxidant activity re- lationships of flavonoids and phenolic acids. Free Radic. Biol. Med. 20 (7), 933–956.
Rodríguez-Nóvoa, et al., 2006. Overview of the pharmacogenetics of HIV therapy. Pharm. J. 6 (4), 234–245.
Sagar, A., Mohanty, A.P., Bahal, A., 2010. Zidovudine-induced myopathy: a study in Indian patients. J. Neurosci. Rural Pract. 1 (2), 63–66.
Saint-Marc, et al., 1999. A syndrome of peripheral fat wasting (lipodystrophy) in patients receiving long-term nucleoside analogue therapy. AIDS 13 (13), 1659–1667.
Scalbert, et al., 2002. Absorption and metabolism of polyphenols in the gut and impact on health. Biomed. Pharmacother. 56 (6), 276–282.
Smith, et al., 2017. Beyond the polymerase-γ theory: production of ROS as a mode of NRTI-induced mitochondrial toxicity. PLoS One 12 (11), e0187424.
Simons, et al., 2010. Greater apparent absorption of flavonoids is associated with lesser human fecal flavonoid disappearance rates. J. Agric. Food Chem. 58 (1), 141–147.
Stankov, et al., 2010. Mitochondrial DNA depletion and respiratory chain activity in primary human subcutaneous adipocytes treated with nucleoside analogue reverse transcriptase inhibitors. Antimicrob. Agents Chemother. 54 (1), 280–287.
Stankov, et al., 2008. Zidovudine impairs adipogenic differentiation through inhibition of clonal expansion. Antimicrob. Agents Chemother. 52 (8), 2882–2889.
Summa, et al., 2008. Discovery of raltegravir, a potent, selective orally bioavailable HIV-integrase inhibitor for the treatment of HIV-AIDS infection. J. Med. Chem. 51 (18), 5843–5855.
Sundaram, et al., 2008. Changes in antioxidant profile among HIV-infected individuals on generic highly active antiretroviral therapy in Southern India. J. Infect. Dis. 12, e61–e66.
Tang, et al., 2014. Role of Nrf2 in chronic liver disease. World J. Gastroenterol. 20 (36), 13079–13087.
Tebay, et al., 2015. Mechanisms of activation of the transcription factor Nrf2 by redox stressors, nutrient cues, and energy status and the pathways through which it at- tenuates degenerative disease. Free Radic. Biol. Med. 88 (Pt B), 108–146.
Touzet, O., Philips, A., 2010. Resveratrol protects against protease inhibitor-induced reactive oxygen species production, reticulum stress and lipid raft perturbation. AIDS 24 (10), 1437–1447.
Trifunovic, A., Larsson, N.G., 2008. Mitochondrial dysfunction as a cause of ageing. J. Intern. Med. 263 (2), 167–178.
UNAIDS, 2016. Countries. 〈http://www.unaids.org〉 (Accessed 7 December 2018).
Venoff, et al., 2007. Mitochondral toxicity of tenofovir emtricitabine and abacair alone and in combination with additional nucleoside reverse transcriptase inhibitors. Antivir. Ther. 12 (7), 1075–1085.
Wallace, D.C., 1992. Mitochondrial genetics: a paradigm for aging and degenerative diseases? Science 256, 628–632.
Wallace, D.C., 1999. Mitochondrial diseases in man and mouse. Science 283, 1482–1488. World Health Organisation, 2010. Antiretroviral Therapy for HIV Infection in Adults and

Adolescents. World Health Organisation, Geneva.
World Health Organisation, 2002. Scaling up Antiretroviral Therapy in Resource-limited Settings: Treatment Guidelines for a Public Health Approach. World Health Organisation, Geneva.
Xu, et al., 2010. miR-17* suppresses tumorigenicity of prostate cancer by inhibiting mitochondrial antioxidant enzymes. PLoS One 5 (12), e14356.
Yamaguchi, T., Katoh, I., Kurata, S.I., 2002. Azidothymidine causes functional and structural destruction of mitochondria, glutathione deficiency and HIV-1 promoter sensitization. Eur. J. Biochem. 268, 2782–2788.
Yamanaka, et al., 2007. Novel mutation of human DNA polymerase gamma associated with mitochondrial toxicity induced by anti-HIV treatment. J. Infect. Dis. 195 (10), 1419–1425.
Yuan, L., Kaplowitz, N., 2009. Glutathione in liver diseases and hepatotoxicity. Mol. Asp. Med. 30 (1–2) (29-4).
Zou, et al., 2012. Tissue distribution study of naringin in rats by liquid chromatography-

Appendix 2: Manuscript Draft, 'Potential hepatoprotective effects of naringenin in combination antiretroviral therapy'.

JPP JOURNAL OF Pharmacy and Pharmacology ROYAL PHARMACEUTICAL SOCIETY

Potential hepatoprotective effects of naringenin in combination antiretroviral therapy

Journal:	*Journal of Pharmacy and Pharmacology*
Manuscript ID	Draft
Wiley - Manuscript type:	Review
Date Submitted by the Author:	n/a
Complete List of Authors:	Govender, Khmera; University of KwaZulu-Natal College of Health Sciences Mofo Mato, Edith; University of KwaZulu-Natal College of Health Sciences Nzuza, Sanelisiwe; University of KwaZulu-Natal College of Health Sciences Kometsi, Lereko; University of KwaZulu-Natal College of Health Sciences Hurchund, Rajendraparsad; University of KwaZulu-Natal College of Health Sciences Kumalo, Sinenhlanhla; University of KwaZulu-Natal College of Health Sciences Gumede, Londiwe; University of KwaZulu-Natal College of Health Sciences Owira, Peter
Keywords:	Clinical Efficacy of Natural Products < Drugs from Natural Sources, Molecular and Clinical Pharmacology, Other topics < Drugs from Natural Sources
Abstract:	Objectives The advent of combination Antiretroviral Therapy (cART) incorporating three antiretroviral drugs has reduced morbidity and mortality in HIV infected patients. However, hepatotoxicity remains a common adverse-effect associated with pharmacotherapy of HIV. Grapefruit-derived flavonoid, naringenin could confer hepatoprotective effects in patients on cART. Key findings A narrative review describing our own experience from experimental laboratory work and published clinical evidence on putative benefits of naringenin on the cART-associated hepatotoxicity is hereby presented. Clinical presentations of hepatotoxicity in HIV infected patients on cART range from asymptomatic elevations of plasma transaminase levels to hepatic failure largely due to increased oxidative stress. Naringenin, a natural flavanone, has been reported to possess promising pharmacological benefits, which include hepatoprotection, antioxidant, anti-apoptotic and anti-inflammatory effects. Emerging evidence suggests that these properties are mediated by naringenin's ability to scavenge free radicals and alleviate oxidative stress by enhancing hepatocellular expression of enzymatic and non-enzymatic antioxidants mediated by increased nuclear factor (erythroid-derived 2)-related factor

	2 (Nrf2) protein expression. Nrf2 is known to upregulate hepatic expression of these antioxidants. Conclusion By relieving oxidative stress, naringenin could potentially be a clinically safe and reliable dietary supplement or adjunct therapeutic agent for the attenuation and prevention of cART-induced hepatotoxicity.

Potential hepatoprotective effects of naringenin in combination antiretroviral therapy

Khmera Govender, Edith Pascale Mofo Mato, Sanelisiwe Nzuza, Lereko Kometsi, Rajendraparsad Hurchund, Sinenhlanhla Inocentia Kumalo, Londiwe Mpumelelo Gumede, Peter Owira*

Molecular and Clinical Pharmacology Research Laboratory, Department of Pharmacology, Discipline of Pharmaceutical Sciences, University of Kwazulu-Natal, P.O. Box X5401, Durban, South Africa.

*Corresponding Author: Owira PMO; Tel: Tel: +27 31 2607720; Fax: + 27 31 260 7907 Email: owirap@ukzn.ac.za

Abstract

Objectives

The advent of combination Antiretroviral Therapy (cART) incorporating three antiretroviral drugs has reduced morbidity and mortality in HIV infected patients. However, hepatotoxicity remains a common adverse-effect associated with pharmacotherapy of HIV. Grapefruit-derived flavonoid, naringenin could confer hepatoprotective effects in patients on cART.

Key findings

A narrative review describing our own experience from experimental laboratory work and published clinical evidence on putative benefits of naringenin on the cART-associated hepatotoxicity is hereby presented.

Clinical presentations of hepatotoxicity in HIV infected patients on cART range from asymptomatic elevations of plasma transaminase levels to hepatic failure largely due to increased oxidative stress. Naringenin, a natural flavanone, has been reported to possess promising pharmacological benefits, which include hepatoprotection, antioxidant, anti-apoptotic and anti-inflammatory effects. Emerging evidence suggests that these properties are mediated by naringenin's ability to scavenge free radicals and alleviate oxidative stress by enhancing hepatocellular expression of enzymatic and non-enzymatic antioxidants mediated by increased nuclear factor (erythroid-derived 2)-related factor 2 (Nrf2) protein expression. Nrf2 is known to upregulate hepatic expression of these antioxidants.

Conclusion

By relieving oxidative stress, naringenin could potentially be a clinically safe and reliable dietary supplement or adjunct therapeutic agent for the attenuation and prevention of cART-induced hepatotoxicity.

Key words: Naringenin, HIV, Antiretrovirals, Hepatotoxicity, Oxidative Stress.

Introduction

While there is no curative treatment for Human Immunodeficiency Virus (HIV) infections, the use of combination antiretroviral therapy (cART) has transformed the disease into a chronic and manageable condition, rather than a terminal illness [1]. Since the discovery of antiviral effects of zidovudine (AZT) against HIV in 1987, 6 mechanistic classes have been developed for use in cART [2], (Table 1).

Recent UNAIDS statistics indicate that, currently there are 20.9 million people living with HIV that are on cARV treatment [2]. It has been estimated that between 1995 and 2010, increased access to antiretroviral therapy has averted an estimated 5.2 million Acquired Immune Deficiency syndrome (AIDS)-related deaths in low- and middle-income countries [3]. Current standard treatment guidelines for HIV infections recommend a cARV regiment containing any 2 NRTIs as backbone and any 1 of NNRTIs or PI (ritonavir-boosted) or more recently an integrase/fusion/entry inhibitor as a base, depending on safety, efficacy and tolerability [4-9], (Table 1). However, side-effects associated with chronic cART use, more so hepatotoxicity remains a major concern [9]. With the UNAIDS/WHO recommended '90-90-90" target; where 90% of people infected with HIV will have been diagnosed, treated and viral loads suppressed, respectively by 2020 [10], cases of cART-induced hepatotoxicity will certainly increase. We have previously shown that d4T or AZT are associated with significant hepatotoxicity in experimental laboratory animals without HIV pathogenesis, and that naringin, a citrus fruit derived flavanone reversed this [11]. Hepatoprotective effects of naringenin have similarly been reported in vivo and in vitro by other researchers in experimental conditions [12-16]. However, there are no clinical data on the potential therapeutic roles of naringenin in hepatoprotection in

cART. This review aims to highlight the importance of naringenin as a reasonable therapeutic intervention in the treatment of cART-induced hepatotoxicity.

ARV-associated hepatotoxicity

Hepatotoxicity is one of the leading causes of non-AIDS related deaths and accounts for approximately 13-18% of all-cause mortality in people infected with HIV [17]. A study in Latin America has shown that even though viral infections are the major causes of liver disease, cARV-induced liver failure still accounted for 2.3% of non-AIDS related mortalities in HIV patients [18]. A retrospective study of 6000 HIV-infected patients has reported that 42% of all cause mortalities due to liver failure could be attributed cART toxicities even though co-infection with viral hepatitis may have been a contributing factor [19]. A cohort study in Switzerland attributed 16% of chronically elevated Alanine Aminotransferase (ALAT) to prolonged exposure to cART without hepatitis virus infections [20]. Clinical presentations of cARV-induced hepatotoxicity range from reversible asymptomatic elevations of serum aminotransaminases {ALAT or Aspartate Aminotransaminase (ASAT)} to hepatic decompensation characterised by jaundice, and hypercoagulopathy [21-23]. These pathological manifestations are generally a consequence of cARVs-induced increased oxidative stress leading to bile acid retention, hepatocyte apoptosis, mitochondrial dysfunction, lipid peroxidation, and endoplasmic reticulum stress [23], (Fig. 1). Since most components of cARVs are substrates of hepatocyte cytochrome P450 (CYP450) enzymes, it is generally not easy to ascribe these hepatotoxic effects to a single ingredient [23-25].

The definition of liver injury is still highly contentious but chronic elevation of ALAT above the upper limit clinically is a surrogate marker for liver disease [26] but generally, elevation of ALAT/or ASAT above upper limits of normal range is accepted as a marker of hepatotoxicity [27]. Many drugs induce hepatocyte γ-glutamylaminotransferase (GGT) but this is generally not

92 a reflection of hepatocellular injury unless accompanied by a parallel increase in alkaline
93 phosphatase (ALP), as in cholestatic lesions [23]. Hepatocyte ultrastructural changes
94 characterised by mitochondrial dysfunctions, sinusoidal degeneration, steatosis, hepatocyte
95 apoptosis and liver failure are often the end result of cART [28].

96 However, it is still not clear why some patients on cARV treatment develop hepatotoxicity yet
97 others don't. Female sex, older age, obesity, hepatitis B or C co-infections, high viral load,
98 nadir CD4 cell counts, heart failure and chronic alcohol ingestion have been identified as
99 predisposing factors to cARV-induced hepatotoxicity [29-35]. Lactic acidosis is reportedly more
100 common in women than men treated with d4T [36, 37], suggesting a gender-linked predisposition.

101 Pharmacogenetic (effects of human gene polymorphisms on drug therapeutic effects) studies
102 have showed that genetic predisposition to cART-induced hepatotoxicity is a clinical reality.
103 Some of the confirmed clinically relevant genotype-phenotype associations include,
104 polymorphisms in the *HLA-B* gene and hypersensitivity reactions to abacavir, a homozygous
105 R964C mutation in the *Pol γ* gene, and E1143G *Poly γ* gene polymorphism leading to d4T-
106 associated lactic acidosis and lipodystrophy, respectively [38-41]. Since efavirenz is both a
107 substrate and inducer of CY2B6, *CYP2B6* haplotype *6*6 has been associated with
108 hepatotoxicity in efavirenz-based cART regimens [42-44]. Increased nevirapine hepatotoxicity
109 has similarly been associated with the genotype TT (c.516/rs3745274) in the CYP2B6 gene [45]
110 and with the interaction of HLA-DRB1*0101 and plasma CD4 counts, suggesting involvement
111 of CD4+ T-lymphocyte-dependent immune response [46, 47]. Genome-wide associations
112 between cART therapy and hepatotoxicity have been studied extensively [48]. However, such
113 studies lack the predictive power of large-scale multivariate clinical surveys since they are
114 based on Single Nucleotide Polymorphisms (SNPS) and are to some extent highly contentious.
115 So far, only abacavir hypersensitivity testing by genotyping for HLA-B*5701 has been applied
116 in clinical practice [47]. For pharmacogenetic tests to be applicable in clinical settings, they need

to be based on large-scale randomised double-blind studies involving multi-ethnic groups, be sensitive and specific, and cost-effective [48]. These stringent requirements so far make interpretation of associations between genetic polymorphisms of genes responsible for cART metabolism and transport, and hepatotoxicity extremely difficult to interpret in the interim.

Mechanisms of cART- induced hepatotoxicity

Nucleo-side/-tide Reverse Transcriptase inhibitors (N(t)RTIs)

NRTIs induce hepatotoxicity by competing with innate nucleotide pools at the binding sites on mitochondrial DNA polymerase-γ (mtDNA poly- γ) [49]. Inhibition of this enzyme leads to depletion of mtDNA and consequently to mitochondrial toxicity in hepatocytes. This causes impaired oxidative phosphorylation, reduced ATP production and eventually hepatocyte apoptosis in what is generally referred to as *mtDNA poly-γ hypothesis* [9]. In addition, NRTIs increase oxidative stress and induce lipid peroxidation [49], (Fig 1). Propensity to cause mitochondrial toxicity (inhibition of DNA pol-γ) by NRTIs has been ranked in the decreasing order of magnitude of: zalcitabine (discontinued) > didanosine > stavudine (withdrawn from first-line treatment)>> zidovudine ≥ emtricitabine = abacavir = tenofovir [9, 50]. Zalcitabine, didanosine, and stavudine have much higher affinities for mtDNA poly- γ and impair hepatic mtDNA replication and cause mitochondrial myopathy to a much greater extent [9] hence their diminished therapeutic use. On the other hand, active metabolites of zidovudine, emtricitabine, abacavir and tenofovir show much weaker interaction with mtDNA poly- γ and are therefore less associated with mitochondrial toxicity [50, 51]. Incidences of hepatotoxicity characterised by transient elevation of serum aminotransferases leading to hepatic inflammation, ER stress, lipid accumulation, lactic acidosis and liver failure or even death have been reported in patients taking regiments containing NRTIs [23, 52]. Consequently, stavudine use in cART has been terminated in clinical practice due to its reputation of inducing hepatotoxicity despite its

excellent viral load suppression effects [53]. Impairment of mtDNA poly-γ by stavudine induces lipid peroxidation leading to severe macrovesicular steatosis with hepatomegaly (Fig 1), unlike lamivudine, an L-enantiomer substituted analogue of cytidine, which has been associated with fewer incidences of hepatotoxicity [54, 55].

Non-Nucleoside Reverse Transcriptase Inhibitors (NNRTIs)

Cases of elevated serum ALAT (particularly) and ALP, bile duct obstruction, jaundice, hepatic necrosis, hepatitis and hepatic failures have been reported in patients treated with cART [23, 56]. NNRTI-induced hepatotoxicity is generally due to bile acid transport obstruction in the hepatobiliary tract, thus leading to cholestasis, apoptosis, increased ER stress and ROS production [3, 23, 56, 57], (Fig. 1). Nevirapine and efavirenz have been associated with hepatotoxicity courtesy of immune mediated hypersensitivity reactions [3, 23, 46, 47, 58]. However, compared to NRTIs, NNRTIs generally appear to be less associated with incidences of hepatotoxicity.

Protease Inhibitors (PIs)

Metabolic disturbances including hepatocyte injury due to increased ER stress and disruption of lipid homeostasis have led to the use of PIs as second-line options in cART regimens [59, 60], (Table 1), (Fig 1). PI-induced ER stress activates the expression of sterol regulatory element-binding proteins (SREBPs) causing hepatic steatosis and hepatotoxicity [59]. Ritonavir for example causes apoptosis mediated by caspase-cascade system and is therefore currently used clinically only as a pharmacokinetic enhancer of other PIs to reduce the risk of metabolic disturbances [4, 24]. Saquinavir also has been reported to cause bile acid retention leading to hepatocyte apoptosis [60, 61], (Fig. 1). Furthermore, we have previously shown that PIs are associated with increased ROS production resulting in oxidative damage by increased lipid peroxidation in vitro [62].

The extent of hepatotoxicity of the newer antiretroviral agents used in cART such as enfuvirtide, raltegravir and maraviroc has not been conclusively evaluated clinically. Most side-effects of drugs used in cART have been noticed only post licensing and not during drug development.

cART-induced oxidative stress

ROS is involved in the regulation of cellular functions such as gene expression for growth or apoptosis, signal transduction pathways and defence against invading microorganisms [63].

ROS are highly reactive biological molecules that are capable of oxidizing proteins, DNA and plasma membranes leading to increased oxidative stress, cellular necrosis and apoptosis [64], (Fig. 2). Hepatic parenchymal cells are often targets of ROS following increased lipid peroxidation, damage to cellular proteins and 8-oxoguanine resulting from DNA damage [65]. Kupffer cells, hepatic stellate cells and endothelial cells are potentially more sensitive to oxidative stress-related molecules [61, 63]. Additionally, proinflammatory cytokines such as TNF-α produced by Kupffer cells due to oxidative stress accelerate apoptosis [39]. Furthermore, proliferation and increased collagen synthesis in hepatic stellate cells triggered by lipid peroxidation promote hepatic steatosis [17]. Non-enzymatic (Vitamin C, E, Glutathione) and enzymatic antioxidants (SOD, CAT, GPx and GST) maintain ROS balance (Fig. 2). Vitamins C, E and glutathione directly neutralize ROS to prevent DNA strand damage and oxidation of biological molecules while SOD reduces reactive O_2 to a more stable compound H_2O_2, which is converted to water and oxygen by CAT [64], (Fig 2). Even though the liver has such elaborate antioxidant defence mechanism, increased oxidative stress can still overwhelm this system and cause hepatocellular injury. It should be noted that the extent of liver damage induced by cART may be exacerbated by concomitant alcohol intake, use of other drugs as well as Hepatitis B/C Virus (HBV/C) co-infections [57, 66]. Strategies to ameliorate cART-induced hepatotoxicity and

improve patient compliance are indeed essential [65]. A putative role of dietary flavonoids such as naringenin which has the potential to either directly quench free radicals or boost endogenous antioxidant capacity is therefore, envisaged at this point.

Naringenin metabolism

Naringenin (4′,5,7-trihydroxyflavanone) is a natural flavanone commonly found in the grapefruit, tomatoes and related citrus species. Naringenin/naringin possesses diverse pharmacological properties such as, anti-apoptotic, anti-diabetic, neuroprotective, hepatoprotective, anti-inflammatory, metal chelating, anti-microbial, anti-mutagenic, anti-ploriferative, free radical scavenging, and antioxidant effects [67-77]. The distinct bitter taste of grapefruit juice for example is attributed to naringenin and there is no known naringenin toxicity to humans or experimental animals [78]. Naringenin is derived from its glycone naringin after hydrolysis in the gut by alpha- rhamnosidase and beta-glucosidase enzymes [72, 73] and then metabolised in the enterocytes and hepatocytes to glucuronides and sulphoglucuronides [12, 78]. Due to its lipophilic nature, naringenin enters hepatocytes by transcellular diffusion or active transport mediated by Multidrug Resistance-associated Protein 1 (MRP1) carriers and is extruded by active efflux protein carriers, P-glycoprotein (P-gp) or MRP2 which have high affinity for it [78-80]. Claims that naringenin is a substrate of organic anion transporting protein (OATP) which mediates its hepatic uptake prior to biotransformation have been disputed [79, 80] but there is credible evidence that naringenin could modulate hepatic expression of this protein [79]. However, what is universally established, is that naringenin has extremely low water solubility due to its hydrophobic ring structure [78, 81-83].

Naringenin has three hydroxyl substitutions, which are capable of donating hydrogen to free radicals, thus scavenging ROS, and is stabilized by resonance due to the presence of aromatic, heterocyclic rings and multiple unsaturated bonds [13]. The B ring in the flavonoid structure can

stabilize the hydroxyl, peroxyl and peroxynitrite radicals to form a stable flavonoid radical (Fig 3).

The relationship between 5-OH and 4-oxo plays an integral role in the ability of naringenin to chelate heavy metal compounds [81], (Fig 3 and 4). Naringenin is a more potent antioxidant than naringin due to the sugar moiety in naringin causing steric hindrance of the scavenging group [67, 69], (Fig. 3).

Naringenin and hepatic oxidative stress

Naringenin scavenges ROS, prevents free radicals attack, hence relieves oxidative stress [14, 83], which causes cART-induced hepatotoxicity by damaging cell membranes, oxidizing lipids and proteins [62, 64]. Naringenin has been noted to upregulate hepatic expression of SOD, CAT, GPx and GST enzymes [12, 15, 80] and further increases GSH/GSSG ratio [15]. GSH is therefore an important co-factor of several detoxifying enzymes besides being responsible for scavenging hydroxyl radicals and single oxygen atoms by directly producing thiyl radicals via glutathione peroxidase, or regeneration of antioxidants vitamin E and C to active forms and facilitating amino acid transport across plasma membranes [64].

It has further been proposed that naringenin impairs lipid peroxidation by reducing cell membrane fluidity due to its lipophilic interaction with polar heads of phospholipids and hence prevents cART-associated oxidative damage to hepatocytes [62, 83-86].

Experimental animal studies have reported protective effects of naringenin against heavy metals (arsenic, lead, copper, iron and cadmium), carbon tetrachloride and ethanol hepatic injury [13, 14, 67, 70, 75, 76, 84, 87]. Our laboratory has recently reported that naringin, a naringenin glycone confers hepatoprotection by reducing oxidative stress in experimental laboratory animals treated with zidovudine or stavudine [11]. In these studies, we noted that naringin abrogated depletion of plasma and hepatic SOD and GPx and also reduced MDA

concentrations in animals that were treated with either zidovudine or stavudine similarly to previously published studies [16, 51, 52, 55]. Even though our studies were done in laboratory animals, we are buoyed by the fact that our findings demonstrated the oxidative effects of NRTIs in a virus free environment. We hence posit that naringin/naringenin could be relieving oxidative stress by upregulating the activity of Nuclear factor-erythroid 2 related factor (Nrf2). Indeed, upregulation of Nrf2 expression has been suggested to be one of the mechanisms by which naringin/naringenin exert antioxidant effects [14, 81, 88].

Naringenin and the Nrf2 hypothesis

HIV-1 infection or cARVs are known to impair Nrf2/Antioxidant Response Element (ARE) signalling pathway due to increased oxidative stress [89-91]. Naringenin has been reported to reduce inflammation by suppressing iNOS, COX-2, TNF-α NF-kB, IL-1 and IL-10 and upregulating the anti-oxidative defence proteins HO-1 and Nrf2 [12, 14 87]. In addition, naringenin showed anti-inflammatory effects by maintaining plasma levels of antioxidants in CCl_4-induced liver injury [14, 76]. It has been reported that naringenin is capable of upregulating Nrf2 protein expression, leading to transcriptional activation of detoxification and antioxidant proteins [14, 15, 80, 81]. Inactive Nrf2 in the cytoplasm interacts with Kelch-like ECH-associated protein 1 (Keap1), a substrate of an adaptor for a cl3- containing E3 ubiquitin ligase [92] in the absence of oxidative stress and becomes degraded by ubiquitin-proteosome pathway [93, 94]. Increased oxidative stress activates Nrf2 by MAPK-mediated phosphorylation or by conformational change in Keap1 leading to dissociation from Keap1 and translocation to the nucleus to form a transcriptional complex with musculoaponeurotic fibrosarcoma (Maf) family of proteins [94-96]. This dimer binds to the promoter region of ARE sequences resulting in co-ordinated transcriptional activation ARE-responsive antioxidants such as NAD(P)H:quinone oxidoreductase (NQO1), glutathione-S-transferases (GST), hemeoxygenase-1 (HO-1), glutathione peroxidase (GPx), glutamate-cystein ligase catalytic subunit GCLC), CAT and

SOD [97, 98]. Therefore, activation of Nrf2 may be a novel strategy to attenuate hepatotoxicity since it is vital for the activation of detoxification genes and preventing oxidative stress. Naringenin treatment has been shown to enhance the expression of Nrf2 protein parallel to antioxidant elements [14, 65 81, 87]. Naringin has experimentally been shown to reverse ionising radiation-induced redox imbalance in rat spleen and protect against acute pancreatitis by activating Nrf2/HO-1signalling pathways [99, 100], protect pancreatic β-cells from streptozotocin-induced diabetes in vitro and in vivo by elevating the activity of Nrf2 [101], and also attenuate H_2O_2-induced mitochondrial dysfunction by Nrf2 –depended mechanisms [102]. It is on the basis of these observations that we strongly advocate the use of naringenin supplements as hepatoprotective agents in patients on cART.

Naringenin prevents hepatocyte apoptosis

Oxidative stress is known to cause altered expression and signal transduction of pro-apoptotic and anti-apoptotic proteins [88]. Treatment with naringin reversed ROS-mediated apoptosis by caspase-3,-8 and -9, inhibited expression of proapoptotic markers Bax and Bad in a rodent model [78] and also increased the expression of anti-apoptotic protein Bcl-2 [12, 87, 88].

We have previously shown that naringin improved hepatocyte apoptosis and oxidative stress in vivo by minimizing nuclear damage and reducing formation of phagolysosomes, maintaining mitochondrial population and preventing cytoplasmic condensation, by significantly decreasing the expression of pro-apoptotic protein Bax and significantly elevating the expression of anti-apoptotic protein Bcl-2 following treatment with either zidovudine of stavudine [11]. Our results corroborated by others have shown that naringin reduced hepatic and plasma malondialdehyde (MDA) and carbonyl protein concentrations and increased the activities of MnSOD and glutathione peroxidase in stavudine- or zidovudine-treated animals [103]. Results from our laboratory and others have further suggested that naringin prevented PI-

induced apoptosis by reducing the activities of caspase-3 or 9 in saquinavir- or atazanavir-treated INS cells lines and in vivo, respectively [62, 104]. Hepatoprotective effects of naringin/naringenin have further been shown to be mediated by scavenging of ROS and modulation of the expression of c-glutamylcysteine synthetase, the rate limiting step in the synthesis pathway of GSH [66, 105].

Clinical Perspectives

With the UN goal of 90-90-90 in ending the AIDS epidemic [106], the prevalence of cART-associated hepatotoxicity will certainly increase. Even though our increased understanding of HIV pathogenesis since 1980s has immensely improved clinical outcomes in the pharmacotherapy of HIV, the risks associated with antiretroviral therapy with respect to drug side-effects still loom large. We now have the flexibility of switching drug combinations to choose from in case of adverse-effects. The newer generation antiretroviral drugs are less associated with severe cases of hepatotoxicity but the fear of post licensing discoveries of adverse-effects like happened with their congeners, calls for caution. In this review we suggest that despite these uncertainties, cARVs could be compounded with naringenin to minimize liver toxicity. Many patients who are compliant on cARVs have viral loads suppressed below detection limits and any cases of hepatotoxicity could largely be attributed to antiretroviral therapy and not to HIV pathogenesis, hence the need for urgent intervention. Naringenin has recently been reported to have antiviral properties [107-109], and therefore this strategy could immensely benefit patients with HIV-1, hepatitis B/C co-infections. Large scale clinical studies are suggested.

Conclusion

Even though much of the evidence adduced in this review is largely experimental, there is clear scientific reason to incorporate naringenin into cART to mitigate hepatotocixity which has led

to treatment failures, increased morbidity and mortality in HIV-1 patients on antiretroviral therapy. Naringenin clearly relieves oxidative stress, reduces inflammation, reduces apoptosis and preserves the integrity of hepatocytes by either directly quenching free radicals or boosting endogenous cellular antioxidant capacity. That naringenin has no known toxicity to humans and has capacity to suppress viral replication makes it an ideal candidate in this perspective.

Declarations

Conflict of interests

Authors have no conflict of interest

Acknowledgement

Our research team at the Molecular and Clinical Pharmacology Research Laboratory for the thought stimulating discussions that led to be writing of this manuscript.

References

1. Este JA. Current status and challenges of antiretroviral research and therapy. *Antivir Res* 2010;85:25–33.
2. UNAIDS. Fact sheet - Latest statistics on the status of the AIDS epidemic, global statistics. Available from: http://www.unaids.org/en/resources/fact-sheet 2017 [Accessed 24/04/2018]
3. Apostolova N, Blas-Garcia A, Galindo MJ, et al. Efavirenz: What is known about the cellular mechanisms responsible for its adverse effects? *Eur J Pharmacol* 2017;812:163–173.
4. World Health Organisation. Scaling up antiviral therapy in resource-limited settings: treatment guidelines for a public health approach. *World Health Organisation* 2002. Geneva
5. Gardner K, Hall PA, Chinnery PF et al. HIV treatment and associated mitochondrial pathology: review of 25 years of in vitro, animal, and human studies. *Toxicol Pathol* 2014;42(5):811-22.

6. The-Antiretroviral-Therapy-Cohort-Collaboration. Life expectancy of individuals on combination antiretroviral therapy in high-income countries: a collaborative analysis of 14 cohort studies. *Lancet* 2008;372:293–299.
7. UNAIDS. Global HIV/AIDS response: epidemic update and health sector progress towards universal access. Progress report. Available from: http://www.unaids.org/en/media/unaids/contentassets/documents/unaidspublication/2011/20111130_ua. 2011_[Accessed on 24/04/2018].
8. Cihlar T, Ray AS. Nucleoside and nucleotide HIV reverse transcriptase inhibitors: 25 years after zidovudine. *Antivir Res* 2010;85:39–58.
9. Kakuda TN. Pharmacology of nucleoside and nucleotide reverse transcriptase inhibitor-induced mitochondrial toxicity. *Clin Ther* 2000;22(6):685-708.
10. UNAIDS. 90–90-90 An ambitious treatment target to help end the AIDS epidemic. http://www.unaids.org/en/resources/documents/2014/90-90-90.2014, Accessed March 7th, 2019).
11. Adebiyi OA, Adebiyi OO, Owira PMO. Naringin reverses hepatocyte apoptosis and oxidative stress associated with HIV-1 Nucleotide Reverse Transcriptase Inhibitors-induced metabolic complications. *Nutrients* 2015;10352–10368
12. Karim N, Jiac Z, Zhenga X et al. A recent review of citrus flavanone naringenin on metabolic diseases and its potential sources for high yield-production. *Trends Food Sci Technol* 2018;79:35–54.
13. Yen FL, Wu TH, Lin LT et al. Naringenin-loaded nanoparticles improve the physicochemical properties and the hepatoprotective effects of naringenin in orally-administered rats with CCl_4-induced acute liver failure. *Pharmaceut Res* 2009;26 (4):893-902.
14. Esmaeili MA, Alilou M. Naringenin attenuates CCl_4-induced hepatic inflammation by the activation of an Nrf2-mediated pathway in rats. Clin Exp *Pharmacol Physiol* 2014;41:416–422.
15. Miler M, Zivanovic J, Ajdzanovic V, et al. Citrus flavanones naringenin and hesperetin improve antioxidant status and membrane lipid compositions in the liver of old-aged Wistar rats. *Exp Geront* 2016;84:49-60.
16. Lin HJ, Ku KL, Lin IS et al. Naringenin attenuates hepatitis B virus X protein-induced hepatic steatosis. *BMC Compl Alt Med* 2017;17:505. https://doi.org/10.1186/s12906-017-2019-2.

17. Smith C, Sabin CA, Lundgren JD et al. Factors associated with specific causes of death amongst HIV-positive individuals in the D:A:D Study. *AIDS* 2010;24:537-1548.
18. Belloso WH, Orellana LC, Grinsztejn B et al. Analysis of serious non-AIDS events among HIV-infected adults at Latin American sites. *HIV Med* 2010;11:554-564.
19. Kovari H, Ledergerber B, Battegay M et al. Incidence and risk factors for chronic elevation of alanine aminotransferase levels in HIV-infected persons without hepatitis B or C virus co-infection. *Clin Infect Dis* 2010;50:502-511.
20. Sulkowski MS. Drug-induced liver injury associated with ARV therapy that includes HIV-1 Protease inhibitors. *Clin Infect Dis* 2004;38(2):90–7.
21. Dieterich DT, Robinson PA, Love J et al. Drug-Induced Liver Injury Associated with the Use of Non-Nucleoside Reverse-Transcriptase Inhibitors. *Clin Infect Dis* 2004;38(2):80–9.
22. Ezhilarasan D, Srilekha M, Raghu R. HAART and Hepatotoxicity. *J Appl Pharma Sci* 2017;7(04):220-226.
23. Nunez M. Hepatotoxicity of antiretrovirals: Incidence, mechanisms and management. *J Hepatol* 2006;44:S132–S139.
24. Echeverría P, Bonjoch A, Puig J et al. Significant improvement in triglyceride levels after switching from ritonavir to cobicistat in suppressed HIV-1-infected subjects with dyslipidaemia. *HIV Med* 2017;18(10):782-786.
25. Wu X, Li Y, Peng K et al. HIV protease inhibitors in gut barrier dysfunction and liver injury. *Curr Opin Pharmacol* 2014;19:61-6.
26. Kovari H, Sabin CA, Ledergerber B et al. Antiretroviral drugs and risk of chronic alanine aminotransaminase elevations in human immunodeficiency virus (HIV)-monoinfected persons: The data collection on adverse-events of anti-HIV drugs study. *Open Forum Infect Dis* 2016;3(1):ofw009. doi: 10.1093/ofid/ofw009.
27. AIDS Clinical Trials Group. Table of grading severity of adult adverse experiences. Rockville, MD: Division of AIDS, National Institute of Allergy and Infectious Disease, Bethesda, 1996
28. Chwiki S, Campos MM, McLaughlin ME et al. Adverse effects of antiretroviral therapy on liver hepatocytes and endothelium in HIV patients: An ultrastructural perspective. *Ultrastruct Pathol* 2017;41(2):186-195
29. Bonnet F, Bonarek M, Morlat P et al. Risk factors for lactic acidosis in HIV-infected patients treated with nucleoside reverse-transcriptase inhibitors: a case-control study. *Clin Infect Dis* 2003;36(10):1324-8

30. Currier JS Havlir DV. Complications of HIV disease and therapy. Top HIV Med 2007;15(2):40-7
31. Fleischer R, Boxwell D, Sherman KE. Nucleoside analogues and mitochondrial toxicity. *Clin Infect Dis* 2004;38(8):e79-80
32. Galli M, Ridolfo AL, Adorni F et al Correlates of risk of adipose tissue alterations and their modifications over time in HIV-1-infected women treated with antiretroviral therapy. *Antivir Ther* 2003;8(4):347-54
33. Gervasoni C, Ridolfo AL, Trifirò G et al. Redistribution of body fat in HIV-infected women undergoing combined antiretroviral therapy. *AIDS* 1999;13(4):465-71
34. Lichtenstein KA, Delaney KM, Armon C et al. Incidence of and Risk Factors for Lipoatrophy (Abnormal Fat Loss) in ambulatory HIV-1 infected women. *JAIDS* 2003;32(1):48-56
35. Godwa C, Newcomb CW, Liu Q, et al. Risk of acute liver Injury with antiretroviral therapy by viral hepatitis status. *OFID* 2017 https://doi.org/10.1093/ofid/ofx012
36. Bolhaar MG, Karstaedt AS. A high incidence of lactic acidosis and symptomatic hyperlactatemia in women receiving Highly Active Antiretroviral Therapy in Soweto, South Africa. *Clin Infect Dis* 2007;45(2):254–260
37. John M, Malla S. Hyperlactatemia syndromes in people with HIV infection. *Curr Opin Infect Dis* 2002;15:23-29
38. Hetherington S, McGuirk S, Powell G et al. Hypersensitivity reactions during therapy with the nucleoside reverse transcriptase inhibitor abacavir. *Clin Ther* 2001;23(10):1603-14
39. Bailey CM, Kasiviswanathan R, Copeland CW et al. R964C Mutation of DNA polymerase γ imparts increased stavudine toxicity by decreasing nucleoside analog discrimination and impairing polymerase activity. *Antimicrob Agents Chemother* 2009;53(6):2610–2612
40. Yamanaka H, Gatanaga H, Kosalaraksa P et al. Novel mutation of human DNA polymerase γ associated with mitochondrial toxicity induced by anti-HIV treatment. *J Infect Dis* 2007;195(10); 1419–1425
41. Chiappini F, Teicher E, Saffroy R et al. Relationship between polymerase gamma (POLG) polymorphisms and antiretroviral therapy-induced lipodystrophy in HIV-1 infected patients: A case-control study. *Curr HIV Res* 2009;7(2):244-253

42. Manosuthi W, Sukasem C, Lueangniyomkul A et al. CYP2B6 haplotype and biological factors responsible for hepatotoxicity in HIV-infected patients receiving efavirenz-based antiretroviral therapy. *Int J Antimicriob Agents* 2014;43:292-296
43. Mutlib AE, Chen H, Nemeth GA et al. Identification and characterization of efavirenz metabolites by Liquid Chromatography/Mass Spectrometry and High Field NMR: Species Differences in the Metabolism of Efavirenz. *Drug Metab Dispos* 1999;27:1319-33
44. Ward BA, Gorski JC, Jones DR et al. The cytochrome P450 2B6 (CYP2B6) is the main catalyst of efavirenz primary and secondary metabolism: implication for HIV/AIDS therapy and utility of efavirenz as a substrate marker of CYP2B6 catalytic activity. *J Pharmacol Exp Ther* 2003;306:287-300]
45. Bertrand J, Chou M, Richardson DM, et al. Multiple genetic variants predict steady-state nevirapine clearance in HIV-infected Cambodians. *Pharmacogenet Genomics* 2012;22(12):868-76
46. Martin AM, Nolan D, James I, et al. Predisposition to nevirapine hypersensitivity associated with HLA-DRB1*0101 and abrogated by low CD4 T-cell counts. *AIDS* 2005;19:97-99
47. Tozzi V. Pharmacogenetics of antiretrovirals. *Antivir Res* 2010;85:190-200
48. Petros Z, Lee MT, Takahashi A et al. Genome-wide association and replication study of hepatotoxicity induced by antiretroviral alone or with concomitant anti-tuberculosis drugs. *OMICS* 2017;21(4):207-216
49. Compiled by Committee For Medicinal Products For Human Use. Reflection paper on in vitro investigation of mitochondrial toxicity of anti-HIV nucleoside reverse transcriptase inhibitors. Eur Med Agency 2007;1-9.
50. Venoff N, Setzer B, Melkaoui K, et al. Mitochondrial toxicity of tenofovir, emtricitabine and abacavir alone and in combination with additional nucleoside reverse transcriptase inhibitors. *Antivir Ther* 2007;12(7):1075-1085.
51. Banerjee A, Abdelmegeed MA, Jang S et al. Zidovudine (AZT) and hepatic lipid accumulation: implication of inflammation, oxidative and endoplasmic reticulum stress mediators. *PLOS one* 2013;8(10):1-9.
52. Hoffmann CJ, Fielding KL, Charalambous S, et al. Antiretroviral therapy using zidovudine, lamivudine, and efavirenz in South Africa: tolerability and clinical events. *AIDS* 2008;22(1):67–74.

468 53. World Health Organisation. Antiretroviral therapy for HIV infections in adults and
469 adolescents. World Health Organisation: Geneva, 2010
470 54. Lim SE, Copeland WC. Differential incorporation and removal of antiviral
471 deoxynucleotides by human DNA polymerase gamma. *J Biol Chem*
472 2001;276(26):23616-23.
473 55. Igoudjil A, Massart J, Begriche K et al. High concentrations of stavudine impair fatty
474 acid oxidation without depleting mitochondrial DNA in cultured rat hepatocytes.
475 *Toxicol In Vitro* 2008;22(4):887-98.
476 56. Apostolova N, Gomez-Sucerquia LJ, Alegre F, et al. ER stress in human hepatic cells
477 treated with efavirenz: Mitochondria again. *J Hepatol* 2013;59:780–789.
478 57. Paemanee A, Sornjai W, Kittisenachai S et al. Nevirapine-induced mitochondrial
479 dysfunction in HepG2 cells. *Sci Rep* 2017;7(1):9194
480 58. Segamwenge IL, Bernard MK. Acute Liver Failure among Patients on Efavirenz-
481 Based Antiretroviral Therapy. *Case Reports Hepatol.* doi: 10.1155/2018/1270716.
482 eCollection 2018.
483 59. Zhang J, He K, Cai L et al. Inhibition of bile salt transport by drugs associated with
484 liver injury in primary hepatocytes from human, monkey, dog, rat, and mouse. *Chem*
485 *Biol Interact* 2016;255:45-54.
486 60. McRae MP, Lowe CM, Tian X et al. Ritonavir, saquinavir, and efavirenz, but not
487 nevirapine, inhibit bile acid transport in human and rat hepatocytes. *J Pharmacol Exp*
488 *Ther* 2006;318(3):1068-75
489 61. Li S, Tan H, Wang N et al. The Role of Oxidative Stress and Antioxidants in Liver
490 Diseases. *Int J Mol Sci* 2015;16:26087–26124.
491 62. Nzuza S, Ndwandwe DE, Owira PMO. Naringin protects against HIV-1 protease
492 inhibitors-induced pancreatic β-cell dysfunction and apoptosis. *Mol Cell Endocrinol*
493 2016;437:1-10
494 63. Sies H. Oxidative stress: a concept in redox biology and medicine. *Redox Biol*
495 2015;4:180-3
496 64. Arauz J, Ramos-Tovar E, Muriel P. Redox state and methods to evaluate oxidative
497 stress in liver damage: From bench to bedside. *Annal Hepatol* 2016;15(2):160-173
498 65. Ivanov AV, Valuev-Elliston VT, Ivanova ON et al. Oxidative stress during HIV
499 infection: Mechanisms and consequences. *Oxid Med Cell Longev* 2016;ID 8910396

66. Wambani JR, Ogola PE, Arika WM, et al. Anti-retroviral drug hepatotoxicity and risk factors in HIV patients with or without hepatitis B and C: A Review. *Infect Dis Ther* 2015;3(6):332-0877.
67. Adil M, Kandhare AD, Ghosh P et al. Ameliorative effect of naringin in acetaminophen-induced hepatic and renal toxicity in laboratory rats: role of FXR and KIM-1. *Renal Failure* 2016;38(6):1007-1020,
68. Priscilla DH, Jayakumar M, Thirumurugan K. Flavanone naringenin: An effective antihyperglycemic and antihyperlipidemic nutraceutical agent on high fat diet fed streptozotocin induced type 2 diabetic rats. *J Funct Foods* 2015;14:363–73.
69. Jung UJ, Kim HJ, Lee JS et al. Naringin supplementation lowers plasma lipids and enhances erythrocyte antioxidant enzyme activities in hypercholesterolemic subjects. *Clin Nutr*. 2003;22, 561–8.
70. Hermenean A, Ardelean A, Stan M et al. Antioxidant and hepatoprotective effects of naringenin and its β-cyclodextrin formulation in mice intoxicated with carbon tetrachloride: A comparative study. *J Med Food* 2014;17(6):670-7.
71. 'Nyane NA, Tlaila TB, Malefane TG, et al. Metformin-like antidiabetic, cardio-protective and non-glycemic effects of naringenin: molecular and pharmacological insights. *Eur J Pharmacol* 2017;803:103-111
72. Chen R, Qi QL, Wang M et al. Therapeutic potential of naringin: an overview. *Pharmaceut Biol* 2016;54(12):3203-3210.
73. Ribeiro IA, Ribeiro MHL. Naringin and naringenin determination and control in grapefruit juice by a validated HPLC method. *Food Control* 2008;19:432–438.
74. Han Y, Su J, Liu X et al. Naringin alleviates early brain injury after experimental subarachnoid haemorrhage by reducing oxidative stress and inhibiting apoptosis. *Brain Res Bull* 2017;133:42–50.
75. Al-Harbi MS. Hepatoprotective effect and antioxidant capacity of naringenin on arsenic-induced liver injury in rats. *Int J Pharm Pharmaceut Sci* 2016;8(4):103-108
76. Dong D, Xu L, Yin L et al. Naringin prevents carbon tetrachloride-induced acute liver injury in mice. *J Funct Food* 2015;12:179–191.
77. El-desokya AH, Abdel-Rahmanb RF, Ahmed OK et al. Anti-inflammatory and antioxidant activities of naringin isolated from *Carissa carandas* L.: *In vitro* and *in vivo* evidence. *Phytomed* 2018;42:126–134.

78. Kanaze FI, Bounartzi MI, Georgarakis M et al. Pharmacokinetics of the citrus flavanone aglycones hesperetin and naringenin after single oral administration in human subjects. *Eur J Clin Nutr* 2007;61:472–477.
79. Bharti S, Rani N, Krishnamurthy B et al. Preclinical evidence for the pharmacological actions of naringin: A review. *Planta Med* 2014;80:437– 451.
80. Nait CM, Al-Ahmad A, Peluso J et al. Quercetin and naringenin transport across human intestinal Caco-2 cells. *JPP* 2009;61:1473–1483.
81. Hernández-Aquino E, Muriel P. Beneficial effects of naringenin in liver diseases: Molecular mechanisms. *World J Gastroenterol* 2018;24(16):679–1707.
82. Shulman M, Cohen M, Soto-Gutierrez A et al. Enhancement of naringenin bioavailability by complexation with hydroxypropoyl-β-cyclodextrin. *PLoS one* 2011;6(4):e18033.
83. Gasparrini M, Giampieri F, Alvarez Suarez JMA et al. Image from AMPK as a new attractive therapeutic target for disease prevention: The role of dietary compounds AMPK and disease prevention. *Curr Drug Targets* 2016;17:865-889.
84. Jayaraman J, Veerappan M, Namasivayam N. Potential beneficial effect of naringenin on lipid peroxidation and antioxidant status in rats with ethanol-induced hepatotoxicity. *JPP* 2009;61:1383–1390.
85. Arora A, Byrem TM, Nair MG et al. Modulation of Liposomal Membrane Fluidity by Flavonoids and Isoflavonoids. *Arch Biochem Biophys* 2000;373 (1):102–109.
86. Hernández-Aquino E, Zarco N, Casas-Grajales S et al. Naringenin prevents experimental liver fibrosis by blocking TGFβ-Smad3 and JNK-Smad3 pathways. *World J Gastroenterol* 2017;23(24): 4354-4368.
87. Pari L, Gnanasoundari M. Influence of naringenin on oxytetracycline mediated oxidative damage in rat liver. Basic Clin Pharmacol Toxicol 2006;98:456–461.
88. Casas-Grajales S, Muriel P. Antioxidants in liver health. *World J Gastrointest Pharmacol Ther* 2015;6(3):59-72.
89. Staitieh BS, Ding L, Neveu WA et al. HIV-1 decreases Nrf2/ARE activity and phagocytic function in alveolar macrophages. *J Leu Biol* 2017;102(2);517-525
90. Singh MV, Kotla S, Le NT et al. Senescent phenotype induced by p90RSK-NRF2 signaling sensitizes monocytes and macrophages to oxidative stress in HIV-positive individuals. Circulation 2019;139;1199-1216

564 91. Mastrantonio R, Cervelli M, Pietropaoli S et al. HIV-Tat Induces the Nrf2/ARE
565 Pathway through NMDA Receptor-Elicited Spermine Oxidase Activation in Human
566 Neuroblastoma Cells. *PLoS One* 2016;11(2):e0149802
567 92. Jaramillo MC, Jang DD. The emerging role of the Nrf2-Keap1 signaling pathway in
568 cancer. *Genes Dev* 2012;27(20):2179-2191
569 93. Bryan HK, Olayanju A, Goldring CE et al The Nrf2 cell defence pathway: Keap1-
570 dependent and -independent mechanisms of regulation. Biochem Pharmacol
571 2013;85(6);705-717
572 94. Kaspar JW, Niture SK, Jaiswal AK. Nrf2:INrf2 (Keap1) signaling in oxidative stress.
573 *Free Radic Biol Med* 2009;47(9):1304-1309
574 95. Zhang H, Davies KJA, Forman HJ. Oxidative stress response and Nrf2 signaling in
575 aging. *Free Radic Biol Med* 2015 Nov;88(Pt B):314-336
576 96. Chen B, Lu Y, Chen Y et al. The role of Nrf2 in oxidative stress-induced endothelial
577 injuries. *J Endocrinol* 2015;225(3):R83-99
578 97. Reddy SP. The antioxidant response element and oxidative stress modifiers in airway
579 diseases. *Curr Mol Med* 2008;8(5):376-83
580 98. Espinosa-Diez C, Miguel V, Mennerich D et al. Antioxidant responses and cellular
581 adjustments to oxidative stress. *Redox Biol* 2015;6:183-197
582 99. Abdel-Magied N, Shedid SM. The effect of naringenin on the role of nuclear factor
583 (erythroid-derived 2)-like2 (Nrf2) and haem oxygenase 1 (HO-1) in reducing the risk
584 of oxidative stress-related radiotoxicity in the spleen of rats. *Environ Toxicol.* doi:
585 10.1002/tox.22745, 2019
586 100. Li Y Pan Y, Gao L et al. Naringenin Protects against Acute Pancreatitis in two
587 experimental models in mice by NLRP3 and Nrf2/HO-1 pathways. *Mediators*
588 *Inflamm.* https://doi.org/10.1155/2018/3232491. 2018
589 101. Rajappa R, Sireesh D, Salai MB et al. Treatment with naringenin elevates the
590 activity of transcription factor Nrf2 to protect pancreatic β-cells from streptozotocin-
591 induced diabetes in vitro and in vivo. *Front Pharmacol* 2019;9:1562. doi:
592 10.3389/fphar.2018.0156
593 102. de Oliveira MR, Brasil FB, Andrade CMB. Naringenin Attenuates H2O2-
594 Induced Mitochondrial Dysfunction by an Nrf2-Dependent Mechanism in SH-SY5Y
595 *Cells. Neurochem Res* 2017;42(11):3341-3350

596 103. Al-Dosari DI, Ahmed MM, Al-Rejaie SS, et al. Flavonoid naringenin attenuates
597 oxidative stress, apoptosis and improves neurotrophic effects in the diabetic rat retina.
598 *Nutrients* 2017;9(1161):1-13.
599 104. Kapoor R, Kakkar P. Naringenin accords hepatoprotection from streptozotocin
600 induced diabetes in vivo by modulating mitochondrial dysfunction and apoptotic
601 signaling cascade. *Toxicol Reports* 2014;1:569–581.
602 105. Wang C, Fan RQ, Zhang YX et al. Naringenin protects against isoniazid- and
603 rifampicin-induced apoptosis in hepatic injury. *World J Gastroenterol*
604 2016;22(44):9775-9783.
605 106. UNAIDS. HIV treatment. Available from www.unaids.org/en/topic/treatment,
606 2017. [Accessed on 17/08/2018]. 44:S132–S139.
607 107. Hernández-Aquino E, Muriel P. Beneficial effects of naringenin in liver
608 diseases: Molecular mechanisms. *World J Gastroenterol* 2018;24(16):1679-1707
609 108. Goldwasser J, Cohen PY, Lin W et al. Naringenin inhibits the assembly and
610 long-term production of infectious hepatitis C virus particles through a PPAR-
611 mediated mechanism. *J Hepatol* 2011;55(5):963-71
612 109. Khachatoorian R, Arumugaswami V, Raychaudhuri S et al. LDivergent
613 antiviral effects of bioflavonoids on the hepatitis C virus life cycle. *Virology*
614 2012;A433(2):346-55.

615 **List of Tables**

616 **Table 1 Classes of medications currently used clinically to treat HIV infections.**

617 **List of Figures**

618 **Figure 1** Mechanisms by which ARVs induce hepatotoxicity. cART induce oxidative
619 stress which damages/inhibits mitochondrial DNA poly-γ leading to impairment of
620 ETC and generation of more ROS which cause lipid peroxidation. ROS caused by PIs
621 (EFV), increases stress signal of the ER leading to activation of SREBS and enhanced
622 lipotoxicity. Oxidative stress in the inner mitochondrial membrane causes leakage of
623 Cyt c, which in conjunction with PIs promote mitochondrial and hepatocyte apoptosis.
624 NRTIs also promote hydrolysis of triglycerides leading to accumulation of FFA and

steatosis. PIs block bile acid transport and excretion into bile canaliculi leading to hepatic steatosis. ER=Endoplasmic reticulum; LPO = lipid peroxidation; ETC= Electron Transport Chain; Cyt c =Cytochrome c; PIs = Protease inhibitors; NRTIs= Nucleoside Reverse Transcriptase Inhibitors; EFV = efavirenz; FFA = free fatty acids; SREBS = Sterol regulatory element-binding protein; DNA poly-γ= DNA polymerase-γ; ROS= Reactive Oxygen Species; cART= combination antiretroviral therapy.

Figure 2 Hepatic redox homeostasis. ROS components such as molecular oxygen, superoxide, hydroxyl radicals, peroxide radicals and non-radicals such as hydrogen peroxide are produced as by-products of normal aerobic metabolism. These free radicals originate from cytochrome P450 metabolism, Mitochondrial Electron transport Chain (ETC), lipoxygenase or NADPH oxidase activity and are physiologically neutralised by antioxidant defence systems comprising GSH, enzymes SOD, SOD GP-x, Catalase and vitamins E/C or dietary flavonoids to maintain a normal redox state. Homeostatic redox balance between the two maintains normal hepatocyte oxidative state but an imbalance between them may cause either cell survival or apoptosis. ROS = Reactive Oxygen Species; GSH = Glutathione; ETC= Electron Transport Chain; GSH-Px = Glutathione Peroxidase; CAT = Catalase; GST = Glutathione S-transferases.

Figure 3 Chemical structures of naringin and its aglycone naringenin

Figure 4 Antioxidant effects of naringenin in hepatocytes. ROS = Reactive Oxygen Species; GSH = Glutathione; ETC= Electron Transport Chain; GSH-Px = Glutathione Peroxidase; CAT = Catalase; GST = Glutathione S-transferases; Nrf2 = Nuclear factor (erythroid-derived 2)-related factor 2; HO-1 = Heme oxygenase.

Table 1

Classes of ARVs	Drug name	Mechanism of action
Nucleoside/Nucleotide Reverse Transcriptase Inhibitors (NRTIs)	Abacavir (ABC); zidovudine (AZT/ZDV) didanosine (ddI); emtricitabine (FTC) lamivudine (3TC); stavudine (d4T)* tenofovir (TDF)^; zalcitabine (ddC)** ^ nucleotide,** Discontinued, *withdrawn	Counterfeit incorporation mechanism leading to termination of viral cDNA chain elongation
Non-Nucleoside Reverse Transcriptase Inhibitors (NNRTIs)	efavirenz (EFV), etravirine (ETR) nevirapine (NVP); rilpivirine (RPV)	Inhibit viral reverse transcriptase by allosteric hindrance leading to termination of viral cDNA chain elongation
Protease Inhibitors (PIs)	amprenavir (APV); nelfinavir (NfV) atazanavir (ATV); ritonavir (RTV-booster) darunavir (DRV); fosamprenavir (fAPV) saquinavir (SQV); indinavir (IDV) tipranavir (TPV); lopiniavir (LPV)	Inhibit viral proteases, which cleave viral polypeptides into mature functional proteins hence they block viral maturation.
Fusion Inhibitors (FIs)	enfuvirtide (T-20)	Inhibits fusion of the viral envelope with CD4 cell's membrane C-region gp41 sequences
Integrase Inhibitors	raltegravir (RAL)	Block integration of proviral DNA into the CD4 cell chromosomal DNA
Co-Receptor/Entry Inhibitors	maraviroc (MAC)	Chemokine CCR5 receptor antagonist. Blocks entry of the viral RNA into the CD4 cell.

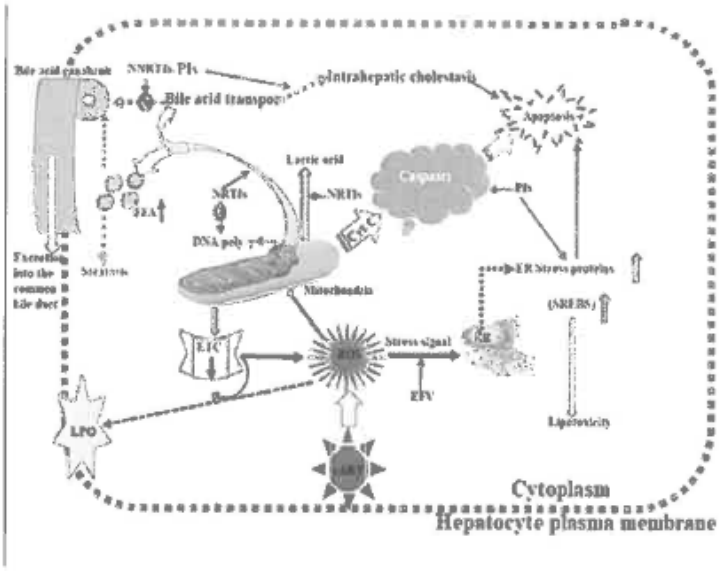


Fig 1

153x109mm (96 x 96 DPI)

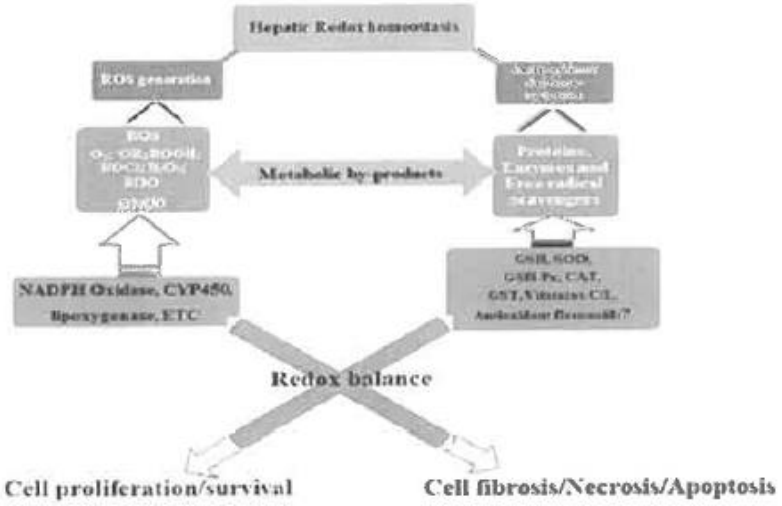


Fig 2

148x100mm (96 x 96 DPI)

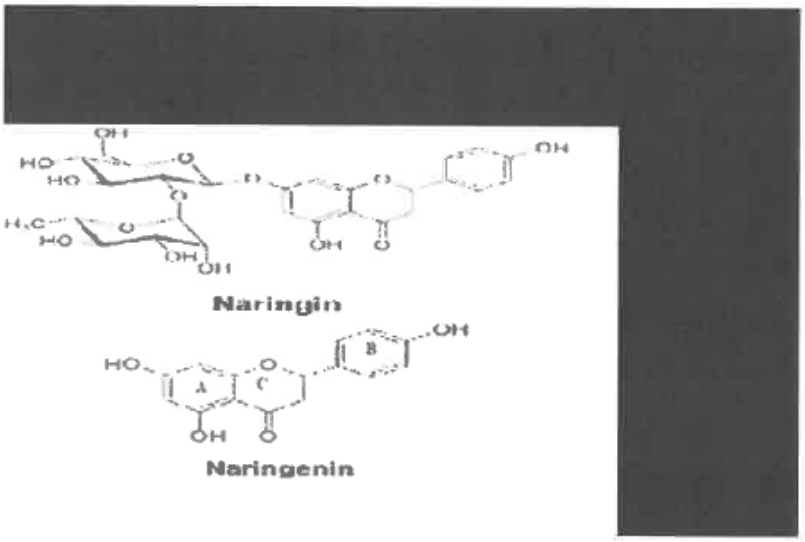


Fig 3

165x109mm (96 x 96 DPI)

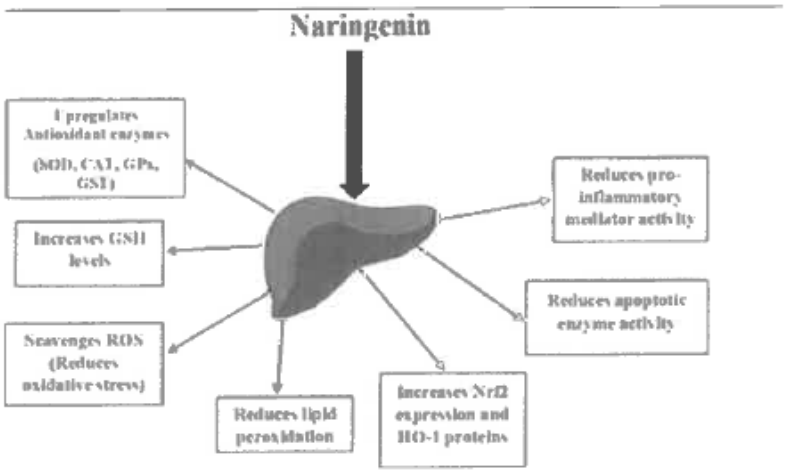


Fig 4

129x76mm (96 x 96 DPI)