Copyright Statement

The digital copy of this thesis is protected by the Copyright Act 1994 (New Zealand).

This thesis may be consulted by you, provided you comply with the provisions of the Act and the following conditions of use:

- Any use you make of these documents or images must be for research or private study purposes only, and you may not make them available to any other person.
- Authors control the copyright of their thesis. You will recognise the author's right to be identified as the author of this thesis, and due acknowledgement will be made to the author where appropriate.
- You will obtain the author's permission before publishing any material from their thesis.

To request permissions please use the Feedback form on our webpage. [http://researchspace.auckland.ac.nz/feedback](http://researchspace.auckland.ac.nz/feedback)

General copyright and disclaimer

In addition to the above conditions, authors give their consent for the digital copy of their work to be used subject to the conditions specified on the [Library Thesis Consent Form](http://researchspace.auckland.ac.nz/feedback) and [Deposit Licence](http://researchspace.auckland.ac.nz/feedback).
The Potential of Combination Drug Therapy for Hepatitis B to Cause Mitochondrial Damage

Keri Tan Garlick (BSc, PGDipSci)

This thesis submitted in fulfilment of the requirements for the degree of Master of Sciences in Pharmacology and Clinical Pharmacology

Department of Pharmacology and Clinical Pharmacology

The University of Auckland

February 2014
ABSTRACT

Even after extensive preclinical and clinical testing, 20% of new drugs will cause adverse drug reactions that will only become apparent after they are on the market. Adverse drug reactions commonly manifest in the liver because it is the primary site for drug metabolism. The leading cause of drug withdrawal is idiosyncratic drug induced liver injury.

Genetic variation and hypersensitivity are long-recognised mechanisms explaining idiosyncratic drug induced liver injury. Mitochondrial toxicity is believed to be an additional mechanism that causes idiosyncratic drug induced liver injury. Mitochondria have a key role in energy production and maintenance of cellular homeostasis. It is also extremely difficult to determine the mechanism of mitochondrial toxicity due to multiple potential targets and their relationship. When one target of mitochondria is impaired it starts a vicious cycle that amplifies damage.

Nucleoside reverse transcriptase inhibitors (NRTIs), used to treat viral infections such as human immunodeficiency virus (HIV) and hepatitis B virus, are known mitochondrial toxins. This has been extensively studied in HIV-positive patients because their most common cause of death is liver failure. It has been shown that using NRTIs in combination increases their mitochondrial toxicity. However, even though NRTI toxicity has been studied in HIV-positive patients, there is limited data for chronic hepatitis B patients. Idiosyncratic drug induced liver injury is often ignored as a potential cause of liver damage due to underlying hepatitis infection. The study of combination drugs used to treat other potential co-morbidities that are common with chronic hepatitis B patients has also not been studied. This present study examines the potential toxicity of cyclosporine, didanosine, lamivudine, and telbivudine in combination using three endpoints for mitochondrial toxicity: mitochondrial DNA, AMP/ADP/ATP, and glutathione levels.
DEDICATION

For Mom and Dad:

Thank you for giving me the ability to focus on my education, the support to study what I am passionate about, and the encouragement to do better than my best.
I would like to thank my supervisor, Dr Malcolm Tingle. Your guidance over this past year has allowed me to develop into an independent thinker and scientist. Not only do I now have the knowledge but also the confidence to enter the world of science.

I would also like to thank the people in the Tingle, Helsby, Paxton, and Cree Labs — Brandi, JP, Jez, Brent, Mike, Jo, Soo Hee, Katie, Elizabeth, and Angel. Your support both in and out of the lab has been valuable to me. Your friendship over this year has made this experience so memorable.

Thanks to Ms Gabriella Blidarean who donated supplies and Dr Lynsey Cree who taught me how to perform qPCR.

Finally, I would like to thank my family, friends, and flatmates. Thank you for always asking how my day was and then listening to me ramble, regardless of whether I had a good or bad day. Without your love and support, I would have never made it through this year.
TABLE OF CONTENTS
ABSTRACT .................................................................................................................. I
DEDICATION ............................................................................................................ II
ACKNOWLEDGEMENTS .......................................................................................... III
LIST OF FIGURES ................................................................................................ VIII
LIST OF TABLES .................................................................................................... VII
ABBREVIATIONS ................................................................................................... IX
1.  INTRODUCTION ................................................................................................... 1
   1.1. Drug induced liver injury .............................................................................. 1
   1.2. Toxicological endpoints .............................................................................. 5
       1.2.1. Cell viability .......................................................................................... 5
       1.2.2. Mitochondrial DNA .............................................................................. 5
       1.2.3. Energy production ................................................................................ 6
       1.2.4. Glutathione .......................................................................................... 8
   1.3. Drugs of interest .......................................................................................... 11
       1.3.1. Nucleoside reverse transcriptase inhibitors ......................................... 11
       1.3.2. Cyclosporine ......................................................................................... 21
   1.4. Hepatitis B virus .......................................................................................... 23
   1.5. Potential co-diseases .................................................................................... 24
       1.5.1. Human immunodeficiency virus .......................................................... 24
       1.5.2. Liver Transplants .................................................................................. 25
   1.6. Summary ...................................................................................................... 26
2.  METHODS ............................................................................................................. 27
   2.1. Materials and equipment ............................................................................ 27
       2.1.1. Materials .............................................................................................. 27
       2.1.2. Equipment ............................................................................................ 28
   2.2. Cell Culture .................................................................................................. 28
       2.2.1. The effects of short-term exposure to high drug concentrations in HepG2 cells 29
2.2.2. The effects of twenty day exposure to clinically relevant concentrations in HepG2 cells 30

2.3. Cell viability assay ..........................................................30

2.4. Determination of human nuclear genes, B2M and GAPDH, and human mitochondrial genes, ND1 and ND4, levels in HepG2 cell lysates by qPCR analysis ..............31

2.4.1. Production of human nuclear genes B2M and GAPDH and human mitochondrial sections B and F, long template by PCR .................................................................31

2.4.2. Determination of mitochondrial DNA level in HepG2 cells by quantitative real time polymerase chain reaction .................................................................34

2.5. Determination of AMP, ADP, and ATP levels in HepG2 cell lysates by HPLC analysis 34

2.6. Determination of reduced glutathione levels in HepG2 cell lysates by HPLC analysis 39

2.7. Statistical methods ..................................................................42

2.7.1. t-test ...........................................................................42

3. RESULTS ...............................................................................43

3.1. Optimisation of literature methods ............................................43

3.1.1. Cell lysis preparation for the mitochondrial DNA assay ..........43

3.1.2. HPLC assay to determine AMP/ADP/ATP concentration in HepG2 cells45

3.1.3. HPLC assay to determine reduced glutathione concentration in HepG2 cells 48

3.2. The effects of drug concentration and exposure time on cell number ....51

3.3. The effect of short-term exposure of HepG2 cells to drugs at ten-fold maximal drug plasma concentration .................................................................54

3.3.1. The effect on mitochondrial DNA level ..................................54

3.3.2. The effect on AMP/ADP/ATP level .....................................54

3.3.3. The effect on reduced glutathione level .................................56

3.4. The effect of 20-day exposure of HepG2 cells to drugs at twice maximal drug plasma concentration ........................................................................57

3.4.1. The effect on mitochondrial DNA level .................................57
3.4.2. The effect on AMP/ADP/ATP level ......................................................... 61
3.4.3. The effect on reduced glutathione level .............................................. 67

4. DISCUSSION ................................................................................................................. 69
  4.1. Background ............................................................................................................. 69
  4.2. Experimental design ............................................................................................. 69
  4.3. Effects of drug exposure time, concentration, and combination on mitochondrial
       endpoints in HepG2 cells ....................................................................................... 72
  4.4. Effects of 20-day HepG2 culture mitochondrial endpoints .............................. 78
  4.5. Potential improvements .......................................................................................... 80
  4.6. Conclusion ............................................................................................................. 81

APPENDIX ...................................................................................................................... 82
  Appendix 1: Buffers, solutions, and gels ................................................................. 82

REFERENCES ............................................................................................................... 84
LIST OF FIGURES

Figure 1-1 The complex interaction of mitochondrial damage.................................................................4
Figure 2-1 An ethidium bromide stained 2% agarose gel showing the long template DNA fragments of human mitochondrial sections, B and F, which contain genes ND1 and ND4 respectively, and human nuclear genes B2M and GAPDH produced by application with PCR.................................................................32
Figure 2-2 The qPCR calibration curve of human nuclear genes, GAPDH (blue line), and human mitochondrial genes, ND1 (red line), and ND4 (green line).........................................................................................................................33
Figure 2-3 Typical chromatograms at 210 nm (A) and 260 nm (B) of the 5 nmol AMP, ADP, and ATP standard on column (B) with a 200 nmol creatine internal standard (A) on column...............................................................................................36
Figure 2-4 Typical chromatograms at 210 nm (A) and 260 nm (B) of the AMP, ADP, and ATP in a HepG2 cell sample (B) with a 200 nmol creatine internal standard on column (A).........................................................................................37
Figure 2-5 Calibration curve of AMP (A), ADP (B), and ATP (C) in reference to its ratio to creatine. ............38
Figure 2-6 Typical chromatogram of a GSH standard (200 pmol on column) with the cysteine internal standard (200 pmol on column)..............................................................................................................................................40
Figure 2-7 Typical chromatogram of HepG2 cell sample (100 μL) with the added cysteine internal standard (200 pmol)..........................................................................................................................................................41
Figure 2-8 The calibration curve for GSH in reference to its ratio to cysteine. Values show the mean ± SD (N = 5)............................................................................................................................................................................42
Figure 3-1 The effect of proteinase K concentration on the extraction of DNA from HepG2 cells..........................44
Figure 3-2 The comparison between the original protocol (127) (red) and the altered protocol (blue)...............46
Figure 3-3 The effects of the volume preparation of HepG2 cells to AMP, ADP, and ATP levels..........................47
Figure 3-4 The preliminary GSH standard curve to determine the linear range......................................................48
Figure 3-5 The effect of incubation time on the detection of GSH......................................................................49
Figure 3-6 Effect of freeze-thawing on the stability of bromobimane-cysteine internal standard..........................50
Figure 3-7 The effects of buffer on the cysteine in HepG2 cells...........................................................................50
Figure 3-8 The effect of cell number on GSH determination..............................................................................51
Figure 3-9 The effect of short-term exposure of HepG2 cells to drugs at ten-fold maximal plasma concentration on cell number..........................................................................................................................................52
Figure 3-10 The effect of twenty day exposure of HepG2 cells to drugs at twice maximal plasma concentrations on cell number.........................................................................................................................................53
Figure 3-11 The effect of short-term exposure of HepG2 cells to drugs at ten-fold drugs at maximal plasma concentration on mtDNA level..............................................................................................................54
Figure 3-12 The effect of short-term exposure of HepG2 cells to drugs at ten-fold maximal plasma concentration on AMP (A), ADP (B), and ATP (C) levels.........................................................................................................................55
Figure 3-13 The effect of short-term exposure of HepG2 cells to drugs at ten-fold maximal plasma concentration on GSH level.........................................................................................................................................................56
Figure 3-14 The effect of long-term exposure of HepG2 cells to drugs at twice the maximal drug plasma concentration on mtDNA level. The mtDNA levels were determined by measuring ND4, a gene on the human mitochondrial genome. This was then taken as a ratio to the measurement of GAPDH, a gene on the human nuclear genome. WN the mean ± SD (N = 3). .................................................................................................................................................57
Figure 3-15 The effect of long-term exposure of HepG2 cells to drugs at twice the maximal plasma concentration on mtDNA level (separated).........................................................................................................................60

VII
List of Figures

Figure 3-16 The effect of long-term exposure of HepG2 cells to drugs at twice the maximal plasma concentration on AMP level. ................................................................. 61
Figure 3-17 The effect of long-term exposure of HepG2 cells to drugs at twice the maximal plasma concentration on AMP level (separated). ................................................................. 62
Figure 3-18 The effect of long-term exposure of HepG2 cells to drugs at twice the maximal plasma concentration on ADP level. ................................................................. 63
Figure 3-19 The effect of long-term exposure of HepG2 cells to drugs at twice the maximal plasma concentration on ADP level (separated). ................................................................. 64
Figure 3-20 The effect of long-term exposure of HepG2 cells to drugs at twice the maximal plasma concentration on ATP level. ................................................................. 65
Figure 3-21 The effect of long-term exposure of HepG2 cells to drugs at twice the maximal plasma concentration on ATP level (separated). ................................................................. 66
Figure 3-22 The effect of long-term exposure of HepG2 cells to drugs at twice the maximal plasma concentration on GSH level. ................................................................. 67
Figure 3-23 The effect of long-term exposure of HepG2 cells to drugs at twice the maximal plasma concentration on GSH (separated). ................................................................. 68

List of Tables

Table 2.1 Drug combinations and their abbreviated letter code. ................................................................. 29
Table 2.2 Maximal drug plasma concentrations ($C_{\text{max}}$) values for cyclosporine, didanosine, lamivudine and telbivudine and calculated two-fold and ten-fold concentrations (84, 124, 125). ................................................................. 29
# ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACN</td>
<td>Acetonitrile</td>
</tr>
<tr>
<td>ADR</td>
<td>Adverse drug reaction</td>
</tr>
<tr>
<td>ADP</td>
<td>Adenosine diphosphate</td>
</tr>
<tr>
<td>AIDS</td>
<td>Acquired immunodeficiency syndrome</td>
</tr>
<tr>
<td>ALT</td>
<td>Alanine aminotransferase</td>
</tr>
<tr>
<td>AMP</td>
<td>Adenosine monophosphate</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>CHB</td>
<td>Chronic hepatitis B</td>
</tr>
<tr>
<td>CK</td>
<td>Creatine kinase</td>
</tr>
<tr>
<td>$C_{\text{max}}$</td>
<td>Maximum drug plasma concentration</td>
</tr>
<tr>
<td>Css</td>
<td>Steady state drug plasma concentration</td>
</tr>
<tr>
<td>CYP450</td>
<td>Cytochrome P 450</td>
</tr>
<tr>
<td>DILI</td>
<td>Drug induced liver injury</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>dNTP</td>
<td>Deoxyribonucleotide</td>
</tr>
<tr>
<td>ETC</td>
<td>Electron transport chain</td>
</tr>
<tr>
<td>EMA</td>
<td>European Medicines Agency</td>
</tr>
<tr>
<td>FAD</td>
<td>Flavin adenine dinucleotide (oxidised)</td>
</tr>
<tr>
<td>FADH$_2$</td>
<td>Flavin adenine dinucleotide (reduced)</td>
</tr>
<tr>
<td>FBS</td>
<td>Foetal bovine serum</td>
</tr>
<tr>
<td>FDA</td>
<td>Food and Drug Administration</td>
</tr>
<tr>
<td>G-6-DP</td>
<td>Glucose-6-phosphate dehydrogenase</td>
</tr>
<tr>
<td>GSH</td>
<td>Glutathione (reduced)</td>
</tr>
<tr>
<td>GSSG</td>
<td>Glutathione (oxidised)</td>
</tr>
<tr>
<td>GPx</td>
<td>Glutathione peroxidase</td>
</tr>
<tr>
<td>GR</td>
<td>Glutathione reductase</td>
</tr>
<tr>
<td>HAART</td>
<td>Highly active antiretroviral therapy</td>
</tr>
<tr>
<td>HBV</td>
<td>Hepatitis B virus</td>
</tr>
<tr>
<td>HCC</td>
<td>Hepatocellular carcinoma</td>
</tr>
<tr>
<td>HCl</td>
<td>Hydrochloric acid</td>
</tr>
<tr>
<td>HIV</td>
<td>Human immunodeficiency virus</td>
</tr>
<tr>
<td>Acronym</td>
<td>Description</td>
</tr>
<tr>
<td>---------</td>
<td>-------------</td>
</tr>
<tr>
<td>HPLC</td>
<td>High-performance liquid chromatography</td>
</tr>
<tr>
<td>HU</td>
<td>Height units</td>
</tr>
<tr>
<td>MEM α</td>
<td>Minimum essential media α</td>
</tr>
<tr>
<td>mtDNA</td>
<td>Mitochondrial deoxyribonucleic acid</td>
</tr>
<tr>
<td>mtRNA</td>
<td>Mitochondrial ribonucleic acid</td>
</tr>
<tr>
<td>NAD</td>
<td>Nicotinamide adenine dinucleotide (oxidised)</td>
</tr>
<tr>
<td>NADH</td>
<td>Nicotinamide adenine dinucleotide (reduced)</td>
</tr>
<tr>
<td>NADPH</td>
<td>Nicotinamide adenine dinucleotide phosphate</td>
</tr>
<tr>
<td>NEM</td>
<td>N-ethylmaleimide</td>
</tr>
<tr>
<td>NRTI</td>
<td>Nucleoside reverse transcriptase inhibitor</td>
</tr>
<tr>
<td>NNRTI</td>
<td>Non-nucleoside reverse transcriptase inhibitor</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>qPCR</td>
<td>Quantitative polymerase chain reaction</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>TAE</td>
<td>Tris-acetate-ethylenediaminetetra acetic acid</td>
</tr>
<tr>
<td>TCA</td>
<td>Trichloroacetic acid</td>
</tr>
<tr>
<td>x g</td>
<td>Gravitational force</td>
</tr>
</tbody>
</table>
1. INTRODUCTION

1.1. Drug induced liver injury

New drugs are required to undergo pre-clinical trials before approval for market sale. Failure in these trials is mainly due to adverse drug reactions (ADRs) (1). Even with preclinical and clinical testing, 20% of new drugs will cause ADRs that only become apparent after a drug is on the market (1) when it is used by a large number of people (2). The occurrence of ADRs has risen greatly since the baby boomer generation due to their increased use of prescription medications (3).

ADRs can be classified into two broad groups: dose-dependent and predictable; and idiosyncratic (4). Predictable ADRs generally occur during a set time frame and all patients are susceptible to them (4). Idiosyncratic ADRs are more difficult to predict because toxicity is a result of a combination of factors such as drug properties, environmental factors, and genetic variation (4). A drug's toxicity can also be the result of unintended off-target activity or non-specific reactions (1). Studying potential toxicity of a drug is complex because there is no 'one size fits all' method (1).

When a new drug causes an ADR that was not observed during testing, it can either be withdrawn from the market or its sales restricted (4). Drug withdrawal is not an ideal situation for pharmaceutical companies because of the high costs of removing a drug from the market and loss of buyer trust and reputation (1). This is also a concern for patients because taking the drug can result in a life-threatening or fatal outcome (5). It is estimated that 6.7% of all hospitalisations are the result of ADRs (6). In Western countries, ADRs are ranked on as the fifth most common cause of inpatient death (6).

ADRs can affect a majority of organs but most commonly manifest in blood cells, liver, and skin (7). The liver is the primary site for drug metabolism, making it a frequent off-target site for drug-induced toxicity (8). A majority of drugs on the market do not cause DILIs (9). However, DILIs are still the leading cause of drug withdrawal (10). The reported incidence is believed to be one in every 10,000 – 100,000 patients (9, 11). Clinical trials normally have a sample size of 2,000 – 5,000 patients; the incidence of DILIs in clinical trials is therefore too small to detect (11). It is also suspected that the incidence of DILIs is underestimated (4) due to underreporting, incomplete observations, or detection/diagnosis difficulties (9).

Hepatotoxicity can result in clinical consequences such as cholestasis, hepatocellular damage, and steatosis (12). In the United States, the leading cause of liver transplant referral in acute liver failure patients is drug-induced hepatotoxicity (2, 9). 10% of acute liver failure is the result of idiosyncratic DILI (4). If a patient with DILI
also has jaundice, there is a 10% chance of fatality (2). Only a quarter of DILI patients will recover spontaneously (2). In the case of drug-induced hepatotoxicity, there are two suggested responses: discontinuation of the suspected drug (9) and/or therapeutic intervention to prevent or relieve toxicity (13).

It is often difficult to determine DILI because its symptoms overlap with those of all known liver diseases (14). In order to diagnose DILI, several criteria must be met. First, the causative medication needs to be identified and the onset of symptoms has to correlate with the suspected medication addition (2). The symptoms of the patient need to correspond with published reports and other potential competing causes need to be ruled out (2). When the drug is removed, the patient’s symptoms should improve (9). If the toxicity is immunological in nature, a re-challenge with the treatment should result in a more severe and rapid toxicity (9). A drug re-challenge with the suspected causative agent is helpful for further confirmation (2). However, this rarely occurs and does not always guarantee a reproducible response (2).

The tests for DILI include liver function tests and histological results from liver biopsies (15). However, liver function tests are limited by the lack of reliable and accurate biomarkers (2). Alanine transaminase (ALT) is a highly sensitive marker; however it is not able to distinguish between DILI or non-hepatic causes (10). For example, ALT activity can be elevated due to metabolic or skeletal muscle disorders (10). Even with elevations in liver enzymes, this may not affect liver function due to its ability to heal from injury (9). A liver biopsy is considered the gold standard for liver diagnosis, but is limited by its invasiveness (16).

Several factors are believed to influence idiosyncratic DILI such as age, cytochrome P450 polymorphism, drug-drug interactions, gender, genetics, immune-mediated responses, lack of adaptation, and nutritional status (10). It is difficult to identify who is at risk due to the low incidence of DILI and limited knowledge on the specific biochemical mechanism of the toxicity (2).

There are a few mechanisms to explain why certain individuals experience idiosyncratic DILI. The first mechanism is that idiosyncratic DILI is potentially a result of genetic polymorphism (5). These genetic polymorphisms can be the result of insertions, deletions, or substitutions in nucleotides and/or variations in the copy number due to deletions or duplications of genes (5). If these genetic polymorphisms occur in locations that encode for drug-metabolising enzymes, transporters, or other proteins related to drug response, it can alter pharmacokinetics, which in turn influences plasma concentrations. A decrease in plasma concentration can result in a loss of clinical effectiveness whereas an increase in plasma concentration can cause an ADR (5).

An additional possible mechanism of ADRs is an immune mediated reaction (7). Some drugs are metabolised to form a reactive intermediate (8). The reactive intermediate can react with a protein and modify it, producing a
hapten which can be processed by antigen-presenting cells (17). They in turn present the antigen to the major histocompatibility complex I on T helper cells, activating an adaptive immune response (17). There is often a delay with immune mediated ADRs, but if a patient is re-challenged with the specific drug, it often results in a more rapid response (7).

A more recent mechanism for idiosyncratic DILI has been identified as drug-induced mitochondria toxicity (18). The mitochondrion is a vital organelle because it controls cell life and death (19). It is responsible for producing cellular energy and maintaining apoptotic factors, cellular ion homeostasis, growth, metabolism, and oxidative stress (20). Mitochondrial dysfunction not only disrupts energy production but also cellular maintenance, resulting in downstream damage in tissue and organ function (19). Mitochondrial toxins can interfere with many different mitochondrial factors, which are extremely interlinked and can result in an amplification of damage. This relationship is shown in Figure 1.1. The initial damage to mitochondria may be silent until it reaches a critical point that induces liver damage (21).

One family of drugs known to affect mitochondria is nucleoside reverse transcriptase inhibitors (NRTIs) (22, 23). These drugs are the primary focus in this project. Their clinical use and mechanism of toxicity will be discussed in section 1.3.
Figure 1-1 The complex interaction of mitochondrial damage. This diagram was based on information from references (21, 24 – 32).
1.2. Toxicological endpoints

As mentioned earlier, mitochondrial toxicity is interlinked. In this project, four toxicological endpoints were studied to verify potential additive or synergistic toxicity. This included cell viability, and mitochondrial DNA, reduced glutathione, and AMP/ADP/ATP levels.

1.2.1. Cell viability

The neutral red assay was then used to verify the cell number was consistent between samples. Neutral red is used in vitro to determine the viability of mammalian cells after exposure to toxic agents (33). It was the first non-genotoxicity in vitro assay approved for regulatory chemical evaluation and is now one of the most commonly-used cytotoxicity assays (34). It determines membrane permeability and lysosomal activity (12).

1.2.2. Mitochondrial DNA

The main hypothesis for the mechanism of NRTI toxicity is its ability to inhibit mitochondrial DNA (mtDNA) polymerase-γ (35). This toxicity is believed to be a result of the similarities between polymerase-γ and viral polymerases (36). Polymerase-γ is important because it is the only polymerase found in mitochondria (37). Not only is it responsible for replication of mtDNA, but it also has an exonuclease activity (38).

The mitochondrion has some similar characteristics to a bacterium because it was once a free living oxygen-breathing bacterium that was engulfed by a nucleus-containing eukaryotic host (39). The start of this symbiotic relationship is believed to have occurred 1.5 billion years ago (40) and now neither can survive without the other (39). A eukaryotic cell requires energy produced by mitochondria (39). Mitochondria cannot survive outside of a eukaryotic cell because the ancient host was required to disable some components of the ancient bacteria to prevent over-proliferation (40). Eukaryotes that failed to do so would have been killed (40). mtDNA has many characteristics that make it similar to bacterial DNA. It is circular DNA and does not contain histones (21, 39). It does not contain exons as all its genes are closely spaced together (41). The only section of non-coding sequence is a 1.1 kb displacement-loop, which controls transcription (41).
mtDNA is important because it encodes 37 genes that are not found on the nuclear genome (27), which are required for mitochondrial function (39). This includes thirteen components of the electron transport chain (ETC) and twenty-two for tRNA (21). All other proteins required for mitochondrial function are encoded on the nuclear genome (42). These genes migrated from the original bacterial genome to the nucleus to help tame ancient bacteria into what is now known as mitochondria (40). They are transcribed and translated in cytosol and their protein is imported into mitochondria (43).

Inhibition of polymerase-γ can result in either depletion of mtDNA copy number or mutations (44). Mutations can also arise from reactive oxygen species (ROS) damage since mtDNA is in the site of ROS production and does not have histone protection (21). mtDNA depletion and mutations can result in mitochondrial dysfunction and thus toxicity (27, 44).

Currently, mtDNA is measured using quantitative real-time polymerase chain reaction (qPCR) (45). This involves primers for mtDNA and for nuclear DNA, which serves as a reference point (46). At the end of each PCR cycle, a fluorescent probe binds to double-stranded DNA and each cycle will result in production of more DNA (46). The amount of DNA product produced is reflective of the starting amount of DNA (46).

However, there are some limitations with just looking at mtDNA. mtDNA levels do not always reflect mitochondrial damage because there can be alternations to mitochondrial RNA (mtRNA) levels (45), thus change between mtDNA and mtRNA levels might not be simultaneous (47). Cells can adapt to decreased mtDNA levels by increasing transcription (47). The next important step is translation of mtRNA into protein (41). Once again, mtDNA and mtRNA levels cannot be used to predict mtDNA encoded proteins levels because translation levels can be altered (45). It is also affected by the degradation rate of proteins (48). In this project, the focus was on mtDNA levels in order to observe polymerase-γ inhibition.

### 1.2.3. Energy production

A major function of mitochondria is to convert metabolic fuel into ATP (49). ATP is the cellular energy currency that allows for maintenance of cellular ion homeostasis, growth, oxidative stress, metabolism, and apoptotic factors (20). Electrons from metabolic fuel get donated to NAD⁺ or FAD, producing NADH and FADH₂ respectively (49). NADH and FADH₂ then transfer electrons to the
electron transport chain (ETC) (50), which consists of four complexes (51). These electrons can either be donated to complex I or complex II, and are then transferred to ubiquinol, complex III, cytochrome c, and complex IV (51). When an electron reaches the end of the ETC, it is donated to oxygen, which will react with hydrogen to produce water (51). This results in no ROS release (50). While electrons are getting shuttled through the ETC, protons are being pumped across the inner membrane into the inner membrane space (52), creating an electro-chemical gradient (53). For protons to re-enter the matrix, they need to travel through the $F_1F_0$-ATP synthase to produce ATP (53). This entire process is known as oxidative phosphorylation. This process can respond to ATP demands and rapidly increase ATP production when stimulated (54).

ROS are natural by-products of physiological processes (49). The majority of electrons will complete the ETC and safely combine with hydrogen and oxygen to produce water (50). However, some electrons will escape and be able to react with oxygen to produce superoxide (50). The majority of cellular ROS is produced by mitochondrial respiration (26). It produces ROS five to ten times the rate of biological processes in cytosol or the nucleus (55). Normally mitochondria can cope with ROS production by intracellular antioxidant defences (2) via increasing expression of mitochondrial antioxidant enzymes and mitochondrial biogenesis to ensure the flow of electrons (50). However, oxidative damage can occur if this balance is disrupted (2).

The ETC can get damaged or inhibited in multiple ways. First, proteins of the ETC can be down-regulated. This can be the result of reduction or mutations in mtDNA or changes to transcription, translation, or degradation of mtRNA or proteins (27, 44, 48, 56). However, as mentioned before, a decrease in mtDNA or mtRNA does not always reflect damage because there can be an adaptive response by alternation to transcription or translation levels (18). Damage can also be directly to the ETC, the inner membrane, or the $F_1F_0$ ATP synthase. (54).

If the ETC is damaged or inhibited, it results in a reduction in ATP production and generation of ROS (21). Electrons will accumulate in the upstream section of the ETC (50) and are unable to undergo complete oxygen reduction, thus producing ROS (21, 50, 53). Complex III is the major site that produces superoxide (21). ROS includes a wide range of molecules. ROS can be molecular oxygen such as a hydroxyl radical, hydrogen peroxide, or superoxide (57). It can also be molecular oxygen with a carbon centre such as organic hydroperoxides, peroxyl radicals, and alkoyl radicals (57).
Finally, ROS can be free radicals such as singlet oxygen, peroxynitrite, and hypochlorous acid (57). Since mitochondria are the main producer of ROS, they are at the most risk for becoming damaged (51). Within mitochondria, ROS have an opportunity to interact with mtDNA, ETC, and lipids (18). ROS can promote the transition pore to open via oxidation of thiols (58, 59). ROS damage and the expression of transition pore cause the activation of cell death (25).

Mitochondrial dysfunction is associated with defective ATP production (54), which becomes depleted early during drug-induced toxicity (12). The depletion of ATP will result in cellular dysfunction (54) and when ATP becomes severely reduced it can induce necrosis (40). Damage to the ETC can also result in changes in the membrane potential (60). Finally, damage to the ETC can result in release of cytochrome c, an apoptotic factor (21). A decrease in ATP levels suggests mitochondrial dysfunction and an altered energy state (12). For that reason, ATP is used as a general marker for cytotoxicity (12).

1.2.4. Glutathione

Of the oxygen that is inhaled, 98% will be used by mitochondria (42). Although 97 – 99% of that oxygen will get reduced to water, 1 – 3% will become ROS, such as superoxide, hydrogen peroxide, and hydroxyl radical (55). Production of endogenous ROS is a trade-off of aerobic metabolism (61). A majority of exogenous compounds are not toxic (62). However, metabolism of these compounds can produce unstable intermediates (62). ROS and unstable intermediates can then inflict injury to a cell by damaging lipids, proteins, DNA, and RNA (62, 63). In order to remove ROS, aerobic cells have developed antioxidant defences such as reduced glutathione (GSH) (64). GSH is uniquely important to aerobic cells because they are not present in anaerobic organisms (65). It is a principal antioxidant and a primary defence against oxygen metabolism (62, 66). Depending on the cell type, the mammalian cell GSH concentration can vary between 0.5 – 12 mM (65, 66). The intracellular GSH concentration is important because it determines the buffer capacity for oxidative stress (66).

GSH is not absorbed from diet (67); this low molecular weight compound is instead made in cytosol (68) in a two-step, energy-dependent reaction (62). GSH consists of glutamate, cysteine, and glycine, which is linearly linked by α-peptide and γ-peptide bonds (68). The first and rate-limiting step is the reaction between glutamate and cysteine, which is catalysed by γ-glutamylcysteine synthase.
Cysteine is also a limiting factor since it is rapidly converted to cystine, thus it has low intracellular concentrations (62). Then, GSH synthase catalyses the reaction between glycine and γ-glutamylcysteine (55, 62). Even though all cells make GSH, the majority of the turnover occurs in the kidneys and liver (62).

The sulphhydryl group of a cysteine residue allows it to participate in antioxidant reactions (65, 68, 70). GSH can be an electron donor in enzymatic reactions or react spontaneously with oxidants with slow kinetics (68, 70). GSH is an electron donor in reactions catalysed by the seleno-protein GSH peroxidase (GPx); the most prominent isoform is GPx-1 (64). It is responsible for removing hydrogen peroxide and lipid peroxidases (64). Another enzymatic reaction is glutathione S-transferase, which conjugates GSH to toxic substances (64). Since glutathione S-transferase is composed of two subunits, there are multiple isoforms of this enzyme (62). There is no known enzymatic reaction that detoxifies hydroxyl radicals, which emphasizes the importance of the spontaneous reactivity of GSH and other antioxidant defences such as ascorbate, carotenoids, tocopherol and urate (64). These antioxidants break the oxidation chain (64).

GSH also has an important role in maintaining functional status of proteins, since it is a major source of nucleophilic thiol and thus maintains thiol:disulphide exchange (71). It reacts with sulfenic acid located on regulatory proteins to form an S-glutathionyl adduct (65, 70). GSH concentration can also affect the transcription of genes responsible for cell proliferation, apoptosis and detoxification enzymes (70, 72).

These reactions result in the conversion of GSH to oxidized glutathione (GSSG) (64). GSSG either diffuses out through the cell membrane or is reduced by glutathione reductase (GR) back to GSH with an electron donated from NADPH (64). In an unstressed cell, the GSH/GSSG ratio is dependent on GR activity (68). Virtually all glutathione is found in the reduced form (64). The GSH/GSSG ratio ranges from 1:100 to 1:1000, depending on a cell type (65). Since it is critical that the balance between GSH and GSSG is maintained, there are multiple feedback mechanisms to control the production of GSH (64). For example, glucose-6-phosphate dehydrogenase (G-6-DP) is sensitive to GSSG because it produces NADPH (64). There is also a negative feedback mechanism by GSH, which will complete with cysteine to inhibit γ-glutamylcysteine synthase (73).
Another characteristic of GSH is that it is resistant to intracellular degradation (62). The c-terminal glycine prevents the cleavage of tripeptide from intracellular γ-glutamyltranspeptidase (62). GSH can only be cleaved by γ-glutamyltranspeptidase on the extracellular membrane, which can separate γ-glutamyl moiety from the cysteinyl-glycine conjugate (62). Cysteinyl-glycine is further metabolized by dipeptidase resulting in a cysteinyl conjugate (62). Acetylation of the cysteine produces mercapturic acid (62). The extracellular concentration of GSH is extremely low, which suggests slow excretion and fast metabolism (65).

GSH is found throughout a cell, but one of the most critical locations is in mitochondria (62, 65). Since mitochondria naturally produce ROS, they are also the primary target for oxidative damage (65). Mitochondrial oxidative damage also depends on GSH and GPx because it does not contain catalase, another key hydrogen peroxide detoxification mechanism (62, 70). The GSH pool is important in mitochondrial function and thus overall cell survival because if ROS are not tightly controlled in mitochondria, they can be released and damage the rest of the cell (65). It is GSH depletion in mitochondria, not cytosol, causes oxidative stress and potentially cell death (65). Depletion of mitochondrial GSH can promote the formation of the transition pore (65). GSH depletion can result in damage to cardiolipin, which is associated with cytochrome c in the inner membrane (70). Cardiolipin damage promotes release of cytochrome c, which then acts as an apoptotic factor (70).

Mitochondria do not contain the necessary enzymes required to make GSH (62, 65), hence they need to transport GSH efficiently from cytoplasm (65). 10 – 15% of intracellular GSH is found in mitochondria (62, 65, 70). However, when factoring in mitochondria size, it results in a similar concentration of GSH as in cytosol (70). GSH is transferred with porins and translocases located in mitochondrial outer membrane (65, 70). Since GSH is anionic at physiological pH, it cannot diffuse into the mitochondrial matrix (65, 70). However, it can be taken up against an electrochemical gradient via carriers in the inner membrane such as 2-oxoglutarate carriers and dicarboxylate carriers (70). In the inner membrane space, GSH is an electron donor to membrane bound GPx-4 to reduce hydrogen peroxide (65, 70). It can also be an electron donor for glutathione S-transferase to detoxify other electrophiles and reactive metabolites (65). Finally, as in cytosol, GSH can control activity of proteins, such as limiting the activity of complex I and IV of the ETC, ATPase, and pyruvate dehydrogenase during oxidative stress (55). Once imported, when GSH becomes oxidized to GSSG,
it cannot be exported (70). However, mitochondria do contain GR, so it is able to reduce GSSG (62, 65, 70). Mitochondria also accumulate GSH precursor γ-glutamylcysteine because it has antioxidant functions and does not have negative feedback on GSH synthesis (65).

Since GSH reacts with ROS, its depletion reflects alterations in oxygen tension, which may be a result of oxidative stress (19, 66). GSH depletion clearly affects mitochondria because they become enlarged and degraded (24). Finally, measuring GSH depletion is important because it is a major player in the progression to cell death (19). In fact, 20 – 30% depletion in GSH is enough to impair cellular detoxification, allowing for progression to cellular damage and death (61). GSH can be depleted either by conjugation to electrophiles or inhibition of its biosynthesis (61). During severe oxidative stress, GR will not be able to reduce GSSG to meet GSH demands, thus GSH concentration will decrease (62). GSH can also be depleted if ATP is seriously diminished because the synthesis of GSH requires ATP (62). Enhancing GSH synthesis can compensate for a GSH loss (65).

1.3. Drugs of interest

Four drugs of interest were selected because they have a potential to be mitochondrial toxins. Three drugs are NRTIs: didanosine, lamivudine, and telbivudine. The fourth drug is cyclosporine, an immunosuppressant. This project not only investigates toxicity of these four drugs used individually, but also if there is an additive or synergistic toxicity when used in combination.

1.3.1. Nucleoside reverse transcriptase inhibitors

All NRTIs are analogues of naturally occurring nucleosides or nucleotides (35). NRTIs require activation to the triphosphate form, which occurs by endogenous kinases (74) at the 5’-ribose position (75). This process can vary depending on tissue type and mitotic state (74). Once activated, NRTIs mimic natural nucleosides and can replace them during viral DNA synthesis, resulting in viral DNA chain termination (38). NRTIs are eliminated unchanged in urine (35) thus NRTI dose adjustment is required in patients with renal impairment (76).

NRTIs are highly effective compounds used to treat viral infections, such as hepatitis B virus (HBV) and human immunodeficiency virus (HIV) (77). The 5’-hydroxyl group of deoxyribose allows for incorporation into a growing strand of DNA (50). However, the absent 3’-hydroxyl group prevents the
addition of other nucleotides, thus the termination of replication (50). Differences in both potency and toxicity of NRTIs can be a result of multiple combinations of factors such as uptake, transport, phosphorylation, affinity for polymerases, incorporation into DNA, and degradation (38). Successful treatment needs to both prevent progression of a viral infection and cause no toxicity to mitochondria (78).

The effectiveness of NRTI treatment is determined by markers such as ALT levels, HBV DNA levels, serum HBeAg presence, HIV RNA levels, and liver fibrosis stage (79, 80). Patients should have regular liver tests and be checked for medication compliance (81). In patients infected with HBV, ALT levels may already be elevated at the start of therapy; analysis is instead taken as change from baseline (15). It is important to prevent liver failure because it results in liver transplants or death every 1.1 per 1,000 person years or 1.1 per 100,000 person years (15).

It was believed that the effect of NRTIs on human DNA polymerases was insignificant (76). However, these compounds have serious ADRs such as hepatic steatosis, neuropathy, myopathy, pancreatitis, and lipodystrophy (77). NRTIs are known mitochondrial toxins (22, 23). Like their ability to inhibit with viral DNA replication, they can also affect mtDNA replication (50). NRTIs have activity against mtDNA polymerase-γ due to its structural similarity to viral polymerases (82). The ribose 3'-hydroxyl group on NRTIs prevent any further nucleoside additions, thus inhibiting mtDNA replication and promoting chain termination (75). NRTIs have been shown to decrease mtDNA, deplete mitochondrial-encoded proteins, and alter mitochondrial morphology (77). As dysfunctional mitochondrial proteins increase, mitochondria will lose their ability to perform critical metabolic functions, such as oxidative phosphorylation and oxidation of fatty acids (82). The disruption of oxidative phosphorylation results in leakage of electrons from the ETC, which will result in ROS production (82). ROS can then go on to damage DNA, lipids, and proteins (82). This results in a vicious cycle, which amplifies mitochondrial damage (13). The United States' Food and Drug Administration (FDA) has classified NRTIs with a black box label (35).

NRTIs approved for chronic hepatitis B (CHB) treatment have substantially less potency on polymerase-γ (35). However, polymerase-γ inhibition is unlikely to be the only mechanism of toxicity (74). A 2005 study by McComsey et al found some patients with lipoatrophy did not have reduced mtDNA levels, suggesting an additional mechanism (83). For example the failed CHB NRTI,
Clevudine, showed no inhibition of polymerase-γ and was not incorporated into mtDNA during preclinical trials (76). Conversely, clevudine was pulled during phase III clinical trials due to myopathy in patients who received the treatment for a year (76). Clevudine toxicity is believed to be the result of preferential phosphorylation by mitochondrial thymidine kinase 2, allowing it to accumulate in mitochondria (76). An additional factor mitochondrial toxicity is the exonuclease activity of polymerase-γ, which allows for the removal of incorporated NRTI (38). The efficiency of this removal can influence NRTI toxicity (38).

NRTIs fall into three categories. The first group consistently impair mtDNA replication, inhibit expression of mtDNA encoded proteins, promote lactate production, and increase intracellular lipid levels (84). Didanosine falls into this category (84). The second group does not cause mitochondrial toxicity on its own, but when combined with other NRTIs, can result in toxicity (84). Lamivudine belongs in this category because when combined with zidovudine it enhances the toxicity of zidovudine (84). The third category does not alter mtDNA levels or proteins encoded by mtDNA, but still impair cell growth (84). Zidovudine falls into the third category (84).

Patients that are co-infected with multiple viral infections require combination drug therapy (22). NRTIs share similar mechanisms of toxicity, which can amplify toxicity when used in combination so much so that a combination’s clinical use can be limited (22, 23, 74). NRTIs’ drug-drug interactions can be quite complex because interactions can occur at a systematic level altering drug absorption, distribution, and elimination, and at a cellular level by fluctuating compartmentation of drugs, compartmental metabolism, and membrane transfer (84).

An additional difficulty in studying NRTI-induced hepatotoxicity is that a majority of gathered information is from developing countries that have limited infrastructures to monitor disease progression (85). Ideally, samples would be taken from fat or muscle for NRTI toxicity since they are tissues that reflect damage, but that is impractical and invasive for a patient (83). As an alternative, blood samples are taken to measure mtDNA levels in peripheral blood (83). However this alternative marker is not accurate (83). Studying NRTI-induced toxicity is difficult because there are multiple potential off-sight targets (74), but tissues that are more energy dependent will be more susceptible to NRTI mitochondrial toxicity (13). It can be a very specific combination of tissue type and NRTI (74). One suggested mechanism is the phosphorylation variation in different tissues (74).
An additional complexity in studying ADRs in CHB patients is that a removal of therapy can result in HBV flares because HBV has an opportunity to replicate (86). If patients with suspected NRTI-induced ADRs continue treatment without intervention and they recover, it is suggestive of HBV flare instead of ADR (87). If it is mitochondrial toxicity, thymine therapy should be administered (88).

An additional possible problem with NRTI therapy is the potential for HIV or HBV drug-resistance via mutations in viral polymerases (89). There is a high chance of mutation, so it is important that patients strictly follow their treatment guidelines (89).

The American Association for the Study of Liver Disease advises discontinuation of all non-essential treatments when a patient develops acute liver failure (86). The incidence of NRTI induced mitochondrial toxicity has been declining since the introduction of newer agents (82). They are generally favoured for treatment since they have fewer side effects (35). However, this might not always be possible in certain areas due to limited access to alternative antivirals and costs (85). There is also limited long-term safety data on these newer NRTIs (35). Although mitochondrial toxicity is an inherit risk with NRTIs, they are still clinically used because its efficacy is greater than any other treatment (23, 74).

The liver is a common target for NRTI toxicity, which can present clinically as lactic acidosis or hepatic steatosis (13). The risk of hepatotoxicity is of interest in CHB patients because this treatment is meant to prevent liver injury, not cause it.

1.3.1.1. Didanosine

Didanosine is a nucleoside analogue of deoxyadenosine (82). Due to its low cost, didanosine is still considered a first line treatment in recourse limited settings (90) and is the backbone of highly active antiretroviral therapy (HAART) (91). Early clinical studies proved there was a better response rate with didanosine compared to zidovudine (91). Didanosine is recommended for both treatment naïve patients and those who have failed one or more antiretroviral regimens (91). It has very little plasma protein binding, distributes well into tissues (91), and is mainly eliminated by the kidneys (82).
NRTIs that are generally associated with ADRs are older thymidine analogues, such as didanosine (86). Didanosine toxicity has been associated with hepatic steatosis, lactic acidosis, pancreatitis, peripheral neuropathy (82), and non-cirrhotic portal hypertension (92).

Compared to other NRTIs, didanosine is at high risk for the development of mitochondrial toxicity (82). Its toxicity is believed to be a result of its higher affinity for polymerase-γ (35, 88). The risk of didanosine toxicity increases with dose (90) and when used in combination with other medication (91). A main concern with didanosine in combination is that it will result in an increase in intracellular didanosine concentrations (22). Confirmed problematic combinations include didanosine with stavudine, emtricitabine, tenofovir or ribavirin (22, 84, 91, 93).

The co-administration of didanosine with tenofovir has been shown to increase didanosine plasma concentrations (90), resulting in increased mitochondrial damage (93). Negredo et al undertook a study in 20 patients and found a reduction of didanosine from 400 mg/day to 250 mg/day was able to produce mtDNA recovery. These patients were also on 300 mg/day tenofovir and 400 mg/day nevirapine (93). However, at the end of the fourteen month dose reduction, other mitochondrial parameters did not recover, which included cytochrome c oxidase activity and mitochondrial protein synthesis (93).

1.3.1.2. Lamivudine
In 1998, lamivudine became the first NRTI approved for CHB treatment (2). Lamivudine was a groundbreaking treatment for CHB because it was the first to successfully reduce the progression rate of cirrhosis and risk of hepatocellular carcinoma (HCC) (94). Ruiz-Sancho et al preformed a clinical trial and showed 98% of patients had a significant decrease in serum HBV DNA level, 72% had ALT normalization, and 16% had HBeAg sero-conversion (95). Lamivudine is effective against HBV and HIV which require dosages of 100 mg/day and 300 mg/day respectively (81).

Lamivudine is designed to be a nucleoside analogue of cytosine (95). Lamivudine is renally eliminated by tubular secretion and glomerular filtration, thus patients with renal disease will required lamivudine dose adjustment (82).
In regards to CHB patients co-infected with HIV, they cannot have lamivudine monotherapy because of the risk of HIV resistance (86). An additional concern is the transmission of the lamivudine-resistant HBV strains (96). 10% of new HBV infections in Western countries have lamivudine resistance (96). If a patient is co-infected with CHB and HIV, the lamivudine dose will be 300 mg/day and used in combination with at least two other antivirals (81).

Lamivudine is a suggested treatment for CHB patients waiting for liver transplants in order to prevent graft reinfection (97). Hepatitis B immune globulin is also given to prevent graft reinfection, specifically with lamivudine treatment due to the high risk of lamivudine resistance (97).

Compared to other NRTIs, lamivudine is less hepatotoxic, thus recommended for patients who are at a risk of hepatotoxicity (88). The lack of toxicity is a result of a lower affinity for polymerase-γ and lack of accumulation in the mitochondria (82, 88). The ‘proof-reading’ ability of polymerase-γ is believed to be able to remove integrated lamivudine from replicating mtDNA (83).

Clinical trials showed lamivudine had similar tolerance to a placebo group (2). Pan-Zhou et al showed no change to HepG2 cell proliferation when exposed to lamivudine concentrations up to 50 µM for six days (98). Following 14 days of lamivudine exposure, there was no structural change to mitochondria observed with an electron microscope and no change in mtDNA levels (98). Honkoop et al undertook a six month study examining potential mitochondrial toxicity of lamivudine in 13 patients with CHB (99). They had no changes in the activity of enzymes encoded by mitochondrial genes (99). Overall, this analysis showed lamivudine had no subclinical signs of mitochondrial damage (99).

However, even though lamivudine has been shown to be safe in multiple studies, its combination with other drugs still needs to be examined. There have been rare reported cases of lamivudine toxicity resulting in reversible myopathy and neuropathy in CHB/HIV co-infected patients, but lamivudine’s specific role is unknown (2). There have also been reports of lactic acidosis in patients on lamivudine, but this has only occurred in HIV-positive patients who are also on other antiviral treatments (76).

An example of lamivudine combination toxicity is a study by Gerschenson et al. The authors studied foetal toxicity of zidovudine and lamivudine combination (100). Foetal toxicity is a concern because HIV-positive pregnant mothers are given antivirals to prevent the spread of the infection to the foetus (100). These two drugs were chosen for that study because they are the most commonly used NTRIs
in pregnant women (100). They exposed pregnant *Erthrocebus patas* monkeys to zidovudine during the last 10 weeks of gestation and then lamivudine during the last 4 weeks of gestation (100). The *patas* monkey has the benefit of not being infected with HIV, thus allowing the study of drug induced mitochondrial toxicity without the potential factor of HIV induced mitochondrial damage (75). Pregnant *patas* monkeys were given 86% of the human zidovudine dose and 84% the human lamivudine dose based on body weight (100). Zidovudine alone showed no mtDNA depletion in brain cerebrum and cerebellum (100). However, when it was combined with lamivudine mtDNA levels were depleted by 35% in cerebrum and 28% in cerebellum (100). The zidovudine plus lamivudine combination also showed greater mtDNA depletion in heart and skeletal muscle compared to the zidovudine treatment alone (100). Electron microscope analysis of foetal cardiac and skeletal muscle in the drug combination group showed mild mitochondrial damage (100). This study suggests that zidovudine and lamivudine have an additive effect (100).

Clinically, the first reported case of zidovudine/lamivudine combination toxicity was in 1999 by Blanche *et al*, who reported two deaths of HIV-1-negative children who were exposed prenatally to NRTIs (101). These children showed widespread mitochondrial toxicity at one year of age (101), which alerted the medical community of a potential of mitochondrial toxicity in HIV-negative children who were exposed to NRTIs *in utero* (75).

A large concern for lamivudine treatment is HBV flare as a result of drug resistance (2), which can result in liver failure or death (102). This is a problem not only because it limits the usage of lamivudine, but also other NRTIs do to cross-resistance (94, 96). After a year of lamivudine treatment, 16 – 40% of patients will have lamivudine resistance (95). After five years of lamivudine treatment, 90% of patient will develop lamivudine resistance (95). This is a result of HBV mutations in the polymerase gene that alters the Tyr-Met-Asp-Asp motif and thus a partial loss of its clinical benefit (95, 97). Even though patients develop lamivudine resistance, the removal of lamivudine causes further problems by resulting in HBV flares (81).

From May 2010, the European Medicines Agency (EMA) stopped recommending lamivudine and telbivudine as first line treatments for CHB due to a chance of the development of drug-resistant strains (103). Of NRTIs, entecavir and tenofovir are preferred as a first line treatment because they
have lower rates of resistance (103). However, lamivudine is still the most widely used NRTI for CHB due to its low cost (102).

1.3.1.3. Telbivudine
Telbivudine was approved in 2006 for treatment of CHB at 600 mg/day (2). Telbivudine is an analogue of thymidine (81, 95). Telbivudine has a greater activity against HBV compared to lamivudine and a lower rate of drug resistance development (81). Unlike lamivudine, telbivudine only has activity against HBV DNA polymerase (95). A one-year trial by Lai et al found that 61% of patients on telbivudine monotherapy had undetectable HBV DNA levels by the end of the study (104). This can be as high as 88% in HBeAg-negative patients (94).

Telbivudine has a half-life of 40 hours and slight accumulation, as repeated dosing has 20 – 50% higher steady state drug plasma concentration compared to a single dose (95). Telbivudine has low binding to plasma proteins and it is widely distributed throughout the body (105). It is not metabolized, thus is unlikely to pharmacologically affect or be affected by substrates metabolized by CYP450 (105). Instead, telbivudine is predominately eliminated by passive glomerular filtration, so moderate to severe renal impairment will alter elimination of telbivudine and dose reductions are required (105). If co-administered with drugs that alter renal excretion, it will affect the pharmacokinetics of telbivudine (105).

Lamivudine and telbivudine have a potential for cross resistance so telbivudine is not recommended for patients who have experienced lamivudine failure and vice versa (81). Two years after starting telbivudine treatment, 25% of HBeAg-positive and 11% of HBeAg-negative patients will develop drug resistance (94).

Telbivudine side effects were shown to be similar to lamivudine, but telbivudine does have a higher incidence of elevated serum creatine phosphokinase levels compared to lamivudine (2). 17% of patients on combination therapy of telbivudine and peginterferon α-2a have moderately severe peripheral neuropathy (2).

Not only does telbivudine have no activity against nuclear DNA polymerases, it also has no activity on polymerase-γ (95). In vitro toxicity testing of telbivudine showed no inhibition of human polymerase α,
β or γ at concentrations up to 100 μmol/L (105). Telbivudine up to 10 μmol/L resulted in no mitochondrial toxicity, which was analysed by mitochondrial structure, function, and lactic production (105). There was no observed toxicity in mice or monkeys during preclinical studies using substantially higher concentrations than the predicted human dose (95). In additional, chronic studies in vitro and animal studies showed no dose limiting toxicity (95). Animal testing suggests that telbivudine does not cause carcinogenicity, genotoxicity, reproductive toxicity, or repeated dose toxicity (105).

Although the most frequent adverse reaction reported in these clinical trials was an increase in ALT levels, similar rates were seen in patient controls (95). A three-year telbivudine safety study in the GLOBE trial caused mild and temporary ADRs (106). Of 399 patients, only two required telbivudine cession due to ADRs (106). Mild ADRs included headache, fatigue, nausea, diarrhoea, and nasopharyngitis (106). Patients also experienced elevated creatine kinase (CK) levels (7.5%), myalgia (22%), myositis (0.5%) and muscular weakness (0.5%) (106). Of patients with elevated CK, 13.3% had developed grade 3/4 by week 156 of telbivudine treatment (106). However, grade 3/4 CK elevation did not correlate to musculoskeletal adverse reactions (106). During the three years of study, CK elevation occurred sporadically and in the majority of cases resolved without intervention by the next visit (106).

More recent data shows that out of all NRTIs used to treat CHB, telbivudine was most commonly associated with myopathy development (76). 9 – 12% of patients on telbivudine treatment will experience moderate elevations in CK (107). This ADR is more common in patients with pre-existing subclinical muscle damage (107). The following two examples are case reports of patients who experienced myopathy on telbivudine treatment.

Finsterer et al reported a case report of a 27-year-old, HIV-negative man with hepatitis C who experienced myotoxic effects after starting telbivudine therapy (107). At 24, he was diagnosed with CHB and received lamivudine, adefovir, and tenofovir treatments (107). However, the treatment was short term because the patient had compliance issues (107). The patient had previously recurring elevations in CK levels, but once the patient correctly took his medication, the HBV load was successfully reduced (107). In April 2009, clinicians prescribed telbivudine therapy without testing for potential resistance (107). Within three weeks, the patient started to experience tiredness and
myalgias (107). CK tests relieved a 10 – 15 fold increase and telbivudine was discontinued in June 2009 (107). Eleven days after telbivudine cession, the patient still had reduced tendon reflexes (107). However, he did not want to undergo addition tests to determine if this was a result of myopathy or neuropathy (107).

Wang et al reported a case of a 25-year-old man with CHB being treated with telbivudine (108). The patient was previously treated with lamivudine and adefovir, both of which were removed due to ineffective HBV suppression (108). Six months after starting treatment, the patient experienced progressive myalgia and weakness (108). The patient first observed the weakness in his arms, was unable to shoot a ball, and had difficulty climbing stairs (108). Blood tests showed the presence of HBsAg and elevated CK, aspartate aminotransferase and lactic acid dehydrogenase levels (108). A muscle biopsy showed myositis, but no mitochondrial changes and once telbivudine treatment was removed, the patient’s condition improved (108). The lack of mitochondrial dysfunction suggests that telbivudine associated myopathy could have a different mechanism, such as oxidative stress, depletion of a substrate, or disruption of protein synthesis (108).

Another potentially serious ADR associated with NRTIs is lactic acidosis. Until 2013, there were no published reports of telbivudine monotherapy causing lactic acidosis (109). Jin et al reported the first case of telbivudine-induced lactic acidosis (109). The patient in this case study was a 36-year-old, HIV-negative man from China (109). He suffered from CHB for 13 years with intermittent elevation in ALT levels, which recovered with some symptomatic treatment (109). However, in September 2011 his ALT levels were elevated to 704 U/L, HBV DNA load was $7.0 \times 10^7$ copies/mL, and his serum was positive for HBeAg, HBeAg, and HBcAg. (109) At that point, he was prescribed 600 mg/day of telbivudine (109). In September 2012, the patient developed anorexia, nausea, vomiting, mild muscle pain, and weakness (109). He was admitted to a hospital after repeated nausea and vomiting for 40 days with no reported abdominal pain or headaches (109). The patient did not have a fever or altered consciousness (109). The CK level was elevated to 3683 U/L (109). A normal blood lactate level is 2.5 mmol/L, this patient presented with a blood lactate level of 4.4 mmol/L (109). Local clinicians determined he was suffering from lactic acidosis and telbivudine treatment was discontinued (109). There was no evidence of infection or organ hypoperfusion (109). However, even with treatment cessation, he continued to decline (109). After haemodialysis lactate levels would return to normal,
but would not remain that way for long term (109). A physical exam relieved the patient had grade 4 muscular weakness in his lower limbs (109). Three months after telbivudine cessation, the patient required 16 haemodialysis treatments and glucocorticosteroïd treatment to return blood lactate levels to normal and HBV DNA to an undetectable level (109). No other potential underlying cause of the lactic acidosis was identified, thus the conclusion it was the result of telbivudine therapy (109).

Telbivudine therapy should be stopped if there is suspected myopathy or evidence of lactic acidosis or severe hepatomegaly since those are side effects associated with NTRIs (105). There is a risk of HBV flare when discontinuing telbivudine, but that is a common side effect of all NRTI discontinuations (95). If telbivudine therapy is removed, patients should be monitored for up to six months after discontinuation (105).

1.3.2. Cyclosporine

Cyclosporine has been used clinically since the early 1980s to prevent graft rejection (110, 111) because it is a strong immunosuppressant (112). Transplant recipients are required to be on cyclosporine for the rest of their lives (112). Cyclosporine improves quality of life and survival of transplant patients by decreasing morbidity, graft rejection and hospitalization days (113). Since reinfection of transplanted liver is a major concern, transplant recipients are required to be treated with NRTIs before and after a transplant (102). Now cyclosporine is also being used for autoimmune disease treatment (110, 111).

Cyclosporine is a neutral lipophilic cyclic undecapeptide that was originally isolated from the fungus *Tolypocladium inflatum gams* (113). Cyclosporine inhibits the cytokine signalling pathway that is critical for T-cell activation (111, 113). Intracellular cyclosporine inhibits protein peptidylprolyl cis-trans isomerase of cyclophilin and calcineruin phosphatase (111). The latter is responsible for dephosphorylation and activation of nuclear factors, which stimulate T cells (111). This prevents the downstream production of cytokines responsible to induce growth and proliferation of B-cells and T-cells (111). Cyclosporine also inhibits production of interleukins 1a, 1b and 6, and γ-interferon, and other lymphokines (111). Treatment has improved the survival of HBV related liver transplant patients to over 85% after one year and 75% after five years (114).
However, cyclosporine has a narrow therapeutic window, with under-dosing resulting in organ rejection and over dose resulting in toxicity (115). Both acute and long term toxicity have been observed with cyclosporine (111). Cyclosporine also has a great risk of drug-drug interaction, which can result in toxicity, over-immunosuppression resulting in infection, and under-immunosuppression resulting in graft rejection (110).

Cyclosporine’s side effects are dose-related and include immunological, hepatic, neurological, and renal complications (113). Since toxicity is related to dosage, cyclosporine toxicity can be managed with either by dose adjustment or drug discontinuation (110, 116). Other reported ADRs include increased risk of cardiovascular events, dyslipidemia, gingival hyperplasia, hypertension, hypertrichosis, and malignancies (110). Cyclosporine cardiotoxicity, hepatotoxicity, and nephrotoxicity are believed to be the result of its ability to generate ROS and lipid peroxidation (110, 111). The mechanism of ROS production is still unknown (111), but suggested mechanisms include increasing mitochondrial Ca$^{2+}$ concentration, blocking the permeability transition pore, and altering the ETC (112). This is further supported by the protective role of antioxidants in cases of cyclosporine-induced nephrotoxicity and hepatotoxicity (110, 111).

The most common complication of cyclosporine treatment is kidney dysfunction, which can result in reduced glomerular filtration rate and alteration in transport (112, 113). Acute nephrotoxicity results in renal dysfunction and vasoconstriction (111). Acute toxicity of cyclosporine is reversible with treatment cession, but symptoms of chronic toxicity will often persist (111). The chronic toxicity is a result of structural damage to kidneys such as tubulointerstitial fibrosis and arteriolopathy, which results in irreversible damage and renal failure (111). The majority of patients on long-term cyclosporine treatment have abnormal renal function (113). This is a concern for CHB patients because NRTIs are mainly eliminated unchanged by kidneys; an NRTI dose adjustment is thus required in patients with altered renal function (76).

Although a rare disorder, cyclosporine can induce hepatotoxicity (117), which normally occurs in the first 90 days after transplant (113). Cyclosporine hepatotoxicity is characterized by an increase in alkaline phosphatase and ALT levels, bile salts in the blood, inhibition of protein synthesis, disturbed lipid secretion, hyperbilirubinemia, cholestasis, and hypoproteinemia (110). An increase in bilirubin
and aminotransferase levels can return to normal with a reduction of cyclosporine dose (113). Its hepatotoxicity is especially limiting in patients with liver transplants (112).

CHB patients with liver transplants will need to use these drugs in combination, to not only prevent graft rejection but also reinfection. Since both NRTIs and cyclosporine have a potential to cause hepatotoxicity via different suggested mechanisms, they were selected for this project.

1.4. Hepatitis B virus

It is important to study potential toxicity of these drugs at a cellular level because DILI is difficult to observe in a clinical setting. It is likely that DILI in CHB is under reported because the liver damage is assumed to be a result of the infection and potential drug toxicity is not further investigated. An additional problem in DILI detection in chronic liver disease patients is the underlying fluctuations in serum markers (118). If a CHB patient presents with liver injury, there are multiple potential causes such as drug toxicity, HBV reactivation, development of drug-resistance, or in the case of immune compromised patients, like those with HIV, immune system reactivation (87). To help distinguish the cause of liver injury in CHB patients, various markers are checked, such as whether a patient has anti-HBcIgM elevations which suggest HBV flare (87). If this occurs late during antiretroviral treatment, this suggests drug resistance (87).

An additional reason why NRTIs used to treat CHB should be examined for hepatotoxicity is because CHB patients are at an increased risk for developing DILI (3) since inflammatory stress may contribute to toxicity (4). Inflammation can change tissue homeostasis and alter expression of hepatocyte transporters (4). Inflammatory cells can directly cause damage by releasing ROS or proteases (4). They can also release mediators such as cytokines and eicosanoids to cause damage indirectly (4). If a patient develops DILI and has previous liver disease, they are more likely to have a poorer outcome compared to healthy patients experiencing DILI (10). Even though it is difficult to detect and often ignored as a possibility, it is important to investigate because these drugs that are prescribed to prevent liver damage might also be causing it.
1.5. Potential co-diseases

Potential additive or synergistic toxicity of drug combinations has not been well studied, especially in patients with CHB (35). Other influential factors, such as additional medical co-morbidities, gender, genetics, age, and nutritional status, have not been examined to understand their potential influence (35). NRTIs used to treat HIV and CHB are very similar and on occasion can be used to treat both viral infections. However, even though these drugs are used clinically in combination, the safety studies for drug approval were studied as a monotherapy (84). Venhoff et al studied HepG2 cells looking at various combinations of NRTIs used to treat HIV (84). The authors found that the majority of compounds resulted in an additive or synergistic toxicity (84). This study has not been repeated in NRTIs used to treat CHB. Not only does this project examine the potential toxicity of CHB NRTIs used in combination, but also other potential co-diseases and their treatments.

1.5.1. Human immunodeficiency virus

It is estimated that four million patients worldwide are co-infected with HIV and CHB (119). 10 – 15% of HIV patients are co-infected with CHB (92). This percentage can be as high as 20% in places such as Southeast Asia (79). Co-infection with HIV and HBV is common due to the shared route of transmission and difficulty for HIV-positive patients to clear HBV infections (79). HIV infection has been shown to influence the progression of HBV infection (119): it decreases HBeAg and HBsAg antibodies and efficacy of HBV therapy (119). HIV increases hepatitis flares, rate of progression to CHB, cirrhosis and HCC, risk of antiviral therapy related hepatotoxicity, and incidence of grade 3/4 ALT elevation, treatment discontinuation, and fatty liver disease (87, 119). Compared to mono-infected patients, co-infected patients have higher rates of liver-related illnesses and deaths (86). Patients co-infected with HBV and HIV are also more likely to experience DILI (10).

Since HIV-positive patients now have long-term survival prospects, clinicians, and scientists have been able to study the potential chronic toxicity of antiretroviral treatment (82). The current leading cause of death in HIV-positive patients is liver disease (92). The other clinical manifestations of mitochondrial toxicity include cardiomyopathy, peripheral neuropathy, and myopathy (75, 83). If patients experience hepatotoxicity, this can interrupt HIV therapy and thus increase morbidity and mortality (120). However, a majority of patients starting antiviral therapy that experience toxicity will recover without intervention (86).
Clinicians need to decide whether to treat the HBV infection, the HIV infection, or both (81). Deciding factors include severity of liver disease, likeliness of therapeutic benefit, and risk of ADRs (81). The difficulty continues with picking the drugs for treatment because clinicians need to balance between finding an effective treatment, preventing drug resistance, and not causing toxicity (81).

Patients who are co-infected with HIV and hepatitis are at twofold risk of hepatotoxicity (92), with 2 – 10% needing to interrupt antiviral therapy due to marked liver enzyme elevation or hepatic injury (88). Determining drug toxicity is extremely difficult in CHB/HIV co-infected patients because symptoms could reflect the natural course of the hepatitis infection, immune reconstitution, or a treatment discontinuation flare (92). An additional problem is serum liver enzyme elevation is a common side effect on antiviral therapy, occurring in 14 – 20% of patients starting treatment (88).

One suggested mechanism for the increased risk for hepatotoxicity in CHB/HIV co-infected patients is that HAART allows for immune reconstitution. During active HIV infection, the immune system is suppressed, but effective treatment allows for the immune system to recover (121). However, this also means the immune system is now capable of attacking HBV-infected hepatocytes, thus causing liver damage (119, 121). This liver damage is associated with a decrease in HIV RNA levels and an increase in CD4 cell count (86). Overall, HIV patients who develop liver failure on HAART, regardless of their HBV status, have a poor prognosis (86).

1.5.2. Liver Transplants
The only course of action in patients with end-stage CHB complications is a liver transplant (102). One concern with a liver transplant in patients with CHB is reinfection of the new liver (102). Before the development of antivirals, the occurrence of graft reinfection was estimated to be between 80 – 100% (114). However, since the introduction of NRTI treatment for CHB, the survival of HBV-related liver transplant patients is over 85% after one year and 75% after five years (114). Not only is NRTI treatment prescribed before the transplant, but it needs to continue afterwards to prevent reinfection (102). During this time immunosuppressants will also need to be prescribed to prevent transplant rejection (122,123). However, this can enhance HBV replication, which increases a risk of liver disease progression and early graft loss (122, 123).
Liver transplants are also becoming increasingly common in HIV-positive patients because liver disease is now their leading cause of death (15). Potential combination toxicity should be a concern for HIV-positive transplant patients because both antivirals and immunosuppressants will be required during the lifetime of the patient to prevent the progression to acquired immunodeficiency syndrome (AIDS) and organ rejection, respectively (15, 122, 123).

Regardless of whether a liver transplant is required for end-stage CHB complications, or NRTI induced hepatotoxicity from HIV or CHB treatment, combination therapy will be required — thus potential additive or synergetic toxicity needs to be examined.

1.6. Summary
Cyclosporine, didanosine, lamivudine, and telbivudine are known potential mitochondrial toxins and have a chance to be seen clinically in combination due to common co-morbidities of CHB, HIV and liver transplants. The aim of this project was to determine the possible additive or synergistic toxicity of these drugs to the mitochondria by monitoring cell viability, and mtDNA, AMP/ADP/ATP, and GSH levels.
2. METHODS

2.1. Materials and equipment

2.1.1. Materials

The human hepatoma HepG2 cell line was obtained from the American Type Culture Collection (ATCC HB-8065). HepG2 cells were typed and confirmed externally by CellBank Australia. Falcon® cell culture flasks (25 cm$^2$) were from In Vitro Technologies (Auckland, New Zealand). Minimum Essential Medium α (MEM α) with no nucleosides, 0.5% Tryspin-EDTA (10X) with no Phenol Red, and MicroAMP Optical 384-well plate were from Life Technologies (Auckland, New Zealand). Foetal bovine serum (FBS) was from Moregate Biotech (Hamilton, New Zealand). Cyclosporine, didanosine, lamivudine, telbivudine, AMP, ADP, ATP, creatine, bromobimane, and neutral red were from Sigma-Aldrich (Sydney, Australia). Perchloric acid and hydrochloric acid (HCl) were from Thermo Fisher Scientific (Scoresby, Australia). Sodium phosphate, potassium phosphate, and Tris-HCl were from J.T. Baker Chemical Co. (Phillipsburg, NJ, USA). Potassium carbonate and sodium chloride were from BioLab (Aust) Ltd (Clayton, Australia). Tetrabutyl ammonium was from Waters (Milfore, MA, USA). Reduced glutathione (GSH) was from ICN Biomedicals (Aura, OH, USA). Cysteine was from BDH Chemicals (Poole, England). N-Ethylmaleimide (NEM) was from Acros (Geel, Belgium). Trichloroacetic acid (TCA) and acetonitrile (ACN) were from Scharlau (Barcelona, Spain). Acetic acid was from Global Science (Auckland, New Zealand). 96 Well flat bottom plates were from Beckman Coulter (Auckland, New Zealand).

Long template and short template primers were kindly donated by Dr Lynsey Cree, Department of Obstetrics and Gynaecology, University of Auckland. She designed them for the human nuclear genes GAPDH and B2M, and human mitochondrial genes ND1 and ND4. Primers were ordered from Integrated DNA Technologies (Auckland, New Zealand). Tween® 20 was from Serva (Auckland, New Zealand). Proteinase K, Taq PCR Master Mix and the QIAQuick Gel Extraction Kit was from Qiagen (Australia). SYBR® Green Select Master Mix was from Life Technologies (Auckland, New Zealand).
2.1.2. Equipment

HepG2 cells were grown in a Heracell 150i CO$_2$ incubator and experiments were conducted in Herasafe™ CS, (Thermo Fisher Scientific, USA). HepG2 cell samples in 1.5 mL tubes were centrifuged in a Heraeus Fresco 17 centrifuge (Thermo Fisher Scientific, USA). 96 Well plates and 384 well plates were centrifuged in a Heraus Multifuge X3R Centrifuge (Thermo Fisher Scientific, USA).

HepG2 cells that were lysed for DNA analysis were heated in a Thermomixer Comfort (Eppendorf, USA). Preliminary PCR reactions were performed with a TC-4000 from Techne (UK). Gels were imaged on a Gel Doc EZ Image from BioRad (USA). qPCR reactions were performed on an ABI7900HT Fast Real Time PCR System from Life Technologies (USA).

The neutral red assay was analysed on a SpectraMax Plus 384 Absorbance Microplate Reader from Molecular Devices (USA).

The high performance liquid chromatography (HPLC) system consisted of a Hewlett Packard series 1100 HPLC system (Agilent Technologies, USA), which included the following: degasser, binary pump, automatic liquid sampler, diode array detection, and fluorescence detector. Two columns were used for this project. Samples for GSH analysis were injected onto a 4.6 mm x 15 cm Phenomenex Prodigy 5 ODS2 column. Samples for AMP, ADP, and ATP analysis were injected onto a 4.6 mm x 15 cm ZORBAX 300 Extend-C18 column.

2.2. Cell Culture

Due to the large amount of drug combinations examined, treatments were abbreviated to the following letter code shown in Table 2.1. HepG2 cells were also exposed to twice and ten-fold the published maximal drug plasma concentration ($C_{max}$). The $C_{max}$ concentration is the corresponding concentration at peak steady state plasma levels. This is shown in Table 2.2.
<table>
<thead>
<tr>
<th>Treatment</th>
<th>Code</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>control</td>
</tr>
<tr>
<td>Cyclosporine</td>
<td>C</td>
</tr>
<tr>
<td>Didanosine</td>
<td>D</td>
</tr>
<tr>
<td>Lamivudine</td>
<td>L</td>
</tr>
<tr>
<td>Telbivudine</td>
<td>T</td>
</tr>
<tr>
<td>Cyclosporine, didanosine</td>
<td>CD</td>
</tr>
<tr>
<td>Cyclosporine, lamivudine</td>
<td>CL</td>
</tr>
<tr>
<td>Cyclosporine, telbivudine</td>
<td>CT</td>
</tr>
<tr>
<td>Didanosine, lamivudine</td>
<td>DL</td>
</tr>
<tr>
<td>Didanosine, telbivudine</td>
<td>DT</td>
</tr>
<tr>
<td>Lamivudine, telbivudine</td>
<td>LT</td>
</tr>
<tr>
<td>Cyclosporine, didanosine, lamivudine</td>
<td>CDL</td>
</tr>
<tr>
<td>Cyclosporine, didanosine, telbivudine</td>
<td>CDT</td>
</tr>
<tr>
<td>Cyclosporine, lamivudine, telbivudine</td>
<td>CLT</td>
</tr>
<tr>
<td>Didanosine, lamivudine, telbivudine</td>
<td>DLT</td>
</tr>
<tr>
<td>Cyclosporine, didanosine, lamivudine, telbivudine</td>
<td>CDLT</td>
</tr>
</tbody>
</table>

Table 2.1 Drug combinations and their abbreviated letter code. The colour coding corresponds to the data in the RESULTS.

<table>
<thead>
<tr>
<th>Drug</th>
<th>C&lt;sub&gt;max&lt;/sub&gt; (µM)</th>
<th>2X C&lt;sub&gt;max&lt;/sub&gt; (µM)</th>
<th>10X C&lt;sub&gt;max&lt;/sub&gt; (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cyclosporine</td>
<td>1.6</td>
<td>3.2</td>
<td>16.0</td>
</tr>
<tr>
<td>Didanosine</td>
<td>11.8</td>
<td>23.6</td>
<td>118.0</td>
</tr>
<tr>
<td>Lamivudine</td>
<td>8.3</td>
<td>16.6</td>
<td>83.0</td>
</tr>
<tr>
<td>Telbivudine</td>
<td>14.1</td>
<td>28.1</td>
<td>140.5</td>
</tr>
</tbody>
</table>

Table 2.2 Maximal drug plasma concentrations (C<sub>max</sub>) values for cyclosporine, didanosine, lamivudine and telbivudine and calculated two-fold and ten-fold concentrations (84, 124, 125).

2.2.1. The effects of short-term exposure to high drug concentrations in HepG2 cells

HepG2 cells were grown in MEM α supplemented with 10% FBS at 37°C and 5% CO₂ in 25 cm² cell culture flasks. A total of 10⁶ cells were plated on day 1. Medium was changed on day 3. On day 5, HepG2 cells were at 90% confluence. HepG2 cells were then exposed to drugs at ten-fold the reported C<sub>max</sub> concentration (Table 2.2), either alone or in combination (Table 2.1). After 2.5 hours of drug exposure, medium was removed and cells were washed with sterile phosphate buffered saline (PBS). 1X trypsin (2.5 mL) was added to the flask and left in an incubator for 8 minutes, then MEM α
with 10% FBS (2.5 mL) was added to the flask. HepG2 cells were kept on ice and prepped for analysis. This experiment was only performed once.

2.2.2. The effects of twenty day exposure to clinically relevant concentrations in HepG2 cells

HepG2 cells were grown in MEM $\alpha$ supplemented with 10% FBS at 37°C and 5% CO$_2$ in 25 cm$^2$ cell culture flasks. HepG2 cells were exposed to drugs at twice the reported C$_{\text{max}}$ concentration (Table 2.2) alone or in combination (Table 2.1). Drugs were refreshed when the medium was changed. A total of $10^6$ cells were plated on day 1. Medium was renewed every third day after replating. HepG2 cells were replated on day 5, 10, 15, and 20 by trypsinization, which is when HepG2 cells reached 90% confluence. On days of replating, HepG2 cells were washed with sterile PBS. 1X trypsin (2.5 mL) was added to the flask and left in an incubator for 8 minutes, then, MEM $\alpha$ with 10% FBS (2.5 mL) was added to the flask. An aliquot (1 mL) of the suspended HepG2 cells was replated in a new 25 cm$^2$ flask containing MEM $\alpha$ with 10% FBS (4 mL). The remainder were kept on ice and prepped for analysis. This experiment was only performed once due to the length of the procedure.

2.3. Cell viability assay

Cell viability was determined using protocol based on Borenfreund and Babich (126). On the day HepG2 cells were harvested, the cell suspension (250 µL) was added to a 96-well plate. Cells were centrifuged at 800 x g for 10 minutes. Medium was removed and cells were washed with non-sterile warm PBS (200 µL). Cells were centrifuged again at 800 x g for 10 minutes. PBS was removed and 20 µg/mL neutral red (200 µL) was added. Neutral red solution was prepared the night before and centrifuged at 1165 x g for 5 minutes to precipitate undissolved dye. HepG2 cells were then incubated for 3 hours at 37°C with 5% CO$_2$. HepG2 cells were centrifuged at 800 x g for 10 minutes and neutral red was removed. HepG2 cells were washed twice with non-sterile warm PBS (200 µL) and centrifuged at 800 x g for 10 minutes between each wash to pellet cells. PBS was removed and neutral red desorb (200 µL) was added. The desorb solution consisted of 1 mL of acetic acid in 100 mL of 50% ethanol. The plate was left for 15 minutes and then placed on a plate shaker for an additional 30 minutes. The 96-well plate was analysed on a SpectraMax Plus 384 Absorbance
Microplate Reader to determine absorbance at 540 nm. The desorb solution was used as the blank reference. This experiment was performed in duplicate.

2.4. Determination of human nuclear genes, *B2M* and *GAPDH*, and human mitochondrial genes, *ND1* and *ND4*, levels in HepG2 cell lysates by qPCR analysis

The protocol used was developed and optimised in Dr Lynsey Cree’s laboratory in the Department of Obstetrics and Gynaecology at the University of Auckland. The primers for both the long and short template for the human nuclear genes *GAPDH* and *B2M* and human mitochondrial genes *ND1* and *ND4* were kindly donated by her lab. There were still a few steps that needed to be performed to determine the amount of HepG2 cells and lysis buffer for this assay.

2.4.1. Production of human nuclear genes *B2M* and *GAPDH* and human mitochondrial sections *B* and *F*, long template by PCR

The generation of the long template for human nuclear genes *B2M* and *GADPH* and human mitochondrial sections *B* and *F*, which contain genes *ND1* and *ND4* respectively, were created in order to produce a standard curve. Four PCR master mixes were made, one for each gene. This included the following: autoclaved water (54.4 μL), 10X immolune buffer (8.75 μL), 25 mM MgCl₂ (7 μL), 10 nM long template forward primer (2.2 μL), 10 nM long template reverse primer (2.2 μL), dNTPs (8.75 μL), and Taq polymerase (24 μL). The master mix (24 μL) was added to autoclaved water (1 μL) or DNA template (1 μL). The DNA template was from a human sample that was prepared by Mr Mike Goldthorpe, Department of Molecular Medicine and Pathology, University of Auckland. The PCR protocol for a cycle was denaturing at 94 °C for 45 seconds, annealing at 45 °C for 55 seconds, and extending at 72 °C for 1 minute and 30 seconds. PCR ran for 30 cycles with a final incubation at 72 °C to finish the extension. DNA products were held at 4 °C. Two positive control samples were run to ensure there was enough to extract, along with one negative control. A 2% agarose gel was produced, which consisted of 2 g agarose and 100 mL TAE. The PCR product was then run on a 2% agarose gel in TAE for 1 hour at 100 mV. It was then stained with 0.5 mg/mL ethidium bromide for 15 minutes.
Figure 2-1 An ethidium bromide stained 2% agarose gel showing the long template DNA fragments of human mitochondrial sections, B and F, which contain genes ND1 and ND4 respectively, and human nuclear genes B2M and GAPDH produced by application with PCR. Lane one contains the 1 Kb DNA ladder (Invitrogen). Lanes 6, 10, and 14 are labelled as “skipped” which means PCR product was not added. The * in lane 4 indicates a possible DNA contamination due to leakage from lane 3. All other lanes are labelled with their PCR product. The “+” indicates human DNA template prepared by Mr Mike Goldthorpe was added, acting as the positive control. The “-” indicates autoclaved distilled water replaced the DNA template, acting as the negative control. The red arrows designate the PCR product which contains the long template DNA fragments containing the genes of interest.

The PCR product has the long template DNA fragments containing the genes of interest are indicated by the red arrows (Figure 2.7). Those sections of the gel were cut to extract the DNA fragments. QIAQuick Gel Extraction Kit from Qiagen (USA) was used. Long templates were diluted to produce a concentration range from $10^8$ to $10^2$ ng/µL and run as a qPCR reaction. qPCR was performed in a 384 well plate. Each well had 10 µL consisting of short forward primer (0.5 µL), short reverse primer (0.5 µL), SYBR Green (5 µL), autoclaved water (3 µL), and long template (2 µL). The 384 well plate was centrifuged at 125 x g for 1 minute. The qPCR reaction for one cycle was as follows: stage 1 at 50 °C for 2 minutes, stage 2 at 95 °C for 2 minutes, stage 3 at 95°C for 15 seconds followed by 60 °C for 1 minute, and a dissociation stage at 15 °C for 15 seconds followed by 95 °C for 15 seconds. The qPCR reaction ran for 40 cycles.
Figure 2-2  The qPCR calibration curve of human nuclear genes, GAPDH (blue line), and human mitochondrial genes, ND1 (red line), and ND4 (green line). Values represent the mean ± SD (N = 3).
All samples worked except for B2M, which produced no signal. The dissociation curve was checked to confirm there was no contamination. Standard curves were produced comparing the human nuclear gene GAPDH to the human mitochondrial genes ND1 and ND4 as shown in Figure 2.8. ND4 was selected as the mitochondrial gene since it produced a slightly more linear relationship to nuclear gene, GAPDH.

2.4.2. Determination of mitochondrial DNA level in HepG2 cells by quantitative real time polymerase chain reaction

On the day of harvest, the HepG2 cell suspension (100 μL) was centrifuged at 600 x g at 4°C for 10 minutes. The medium was removed and cells were stored in -80 °C until analysis. At the time of analysis, lysis buffer (50 μL) was added. The lysis buffer consisted of 0.5 ng/mL proteinase K, 0.5% Tween 20, and 50 mM Tris-HCl pH 8.5. Samples were incubated at 55 °C for 16 hours and then the temperature was increased to 95°C for 10 minutes. HepG2 cells were centrifuged for a few seconds to pellet cellular debris and supernatant was taken for PCR analysis. The qPCR was performed in a 384 well plate. Each well had 10 μL consisting of 0.5 μL of short forward primer, 0.5 μL of short reverse primer, 5 μL SYBR® Green, 3 μL autoclaved water, and 2 μL of DNA standard or cell sample. The 384 well plate was centrifuged at 125 x g for 1 minute. The qPCR reaction for one cycle was as follows: stage 1 at 50 °C for 2 minutes, stage 2 at 95 °C for 2 minutes, stage 3 at 95 °C for 15 seconds followed by 60 °C for 1 minute, and a dissociation stage at 15 °C for 15 seconds followed by 95 °C for 15 seconds. The qPCR reaction ran for 40 cycles.

2.5. Determination of AMP, ADP, and ATP levels in HepG2 cell lysates by HPLC analysis

AMP, ADP and ATP were measured using an adapted and optimised protocol version of a previously described HPLC method by Manfredi et al (127).

On the day HepG2 cells were harvested, the HepG2 cell suspension (1 mL) was used to prepare the sample for ATP analysis. Cells were centrifuged at 600 x g for 10 minutes at 4 °C. The medium was removed and ice cold 0.4 M perchloric acid (150 μL) was added. The perchloric acid not only lysed the cells, but also inactivated ATPases. All samples were kept on ice throughout the ATP assay to reduce ATP and ADP degradation. Cell samples were vortexed and left for 30 minutes on ice. The
HepG2 cell lysis was then centrifuged at 17,000 x g for 10 minutes at 4 °C. The supernatant (121.4 μL) was added to 20 mM creatine in 0.4 M perchloric acid (15 μL). This resulted in a creatine internal standard of 200 nmol on column. Such a large amount was used in order to distinguish it from an interfering peak. This internal standard showed a precision of 11% in quality control samples and cell samples. Finally, 4 M potassium carbonate (13.6 μL) was added to basify the solution. This sample was left on ice for 10 minutes and stored in -80°C for at least 1 hour or until analysis to promote the precipitation of the salt.

Instead of doing separate AMP, ADP, and ATP standards, the three compounds were combined. This is because all three compounds were found in HepG2 cells. They were diluted in 0.4 M perchloric acid and followed the HepG2 analysis prep at the addition of the 20 mM creatine internal standard and 4 M potassium carbonate. They were also stored in -80 °C. AMP/ADP/ATP standards included the calibration curve and quality control samples. The quality control had 0.5 nmol, 1 nmol, and 5 nmol on column.

At the time of analysis, samples were thawed at room temperate then immediately centrifuged at 17,000 x g for 10 minutes at 4 °C. The supernatant (100 μL) was injected onto a 4.6 mm x 15 cm ZORBAX 300 Extend-C18 column. Mobile solutions consisted of solution A (25 mM NaH₂PO₄, 100 mg/L tetrabutylammonium, pH 5) and solution B (10% v/v ACN, 200 mM NaH₂PO₄, 100 mg/L tetrabutylammonium, pH4). The gradient was 100% A for 0-5 minutes and 100% A to 100% B from 5-20 minutes. The column was then re-equilibrated from 20 – 31 minutes with 100% A. The eluent was UV monitored at 210 nm for creatine and 260 nm for AMP, ADP, and ATP. Typical chromatograms for a standard sample and cell sample are shown in Figure 2.4 and Figure 2.5 respectively.
Figure 2-3  Typical chromatograms at 210 nm (A) and 260 nm (B) of the 5 nmol AMP, ADP, and ATP standard on column (B) with a 200 nmol creatine internal standard (A) on column. The sample was in 0.4 M perchloric acid basified with 4 M potassium carbonate. The ADP, ATP and creatine show sharp peaks. The AMP peak is slightly split.
Figure 2-4 Typical chromatograms at 210 nm (A) and 260 nm (B) of the AMP, ADP, and ATP in a HepG2 cell sample (B) with a 200 nmol creatine internal standard on column (A). The sample was in 0.4 M perchloric acid basified with 4 M potassium carbonate. The ATP and creatine peaks are easily distinguishable from the background. The AMP and ADP peaks were determined by overlaying with an AMP/ADP/ATP quality control standard. This chromatogram was taken from day 15 CLT HepG2 sample.
A standard curve was constructed between 0 and 5 nmol AMP, ADP and ATP on column, as shown in Figure 2.6. The peak area of ATP was compared with the peak area of creatine in order to create a ratio which was plotted against the corresponding known amount of ATP. This process was repeated for AMP and ADP. Six points were used to make this standard curve, adhering to the FDA and EMA guidelines. The experiment was done in quintuplicate. There was a linear relationship for all three compounds. Following the FDA and EMA guidelines, the calibration standards had ± 15% deviation from nominal concentration and lower limit of quantification standards had ± 20% deviation from nominal concentration.

Figure 2-5 Calibration curve of AMP (A), ADP (B), and ATP (C) in reference to its ratio to creatine. Values show the mean ± SD (N = 5).
2.6. Determination of reduced glutathione levels in HepG2 cell lysates by HPLC analysis

GSH was measured using an adapted and optimised version of a previously described HPLC method by Cotgreave and Moldeus (128). Bromobimane was first dissolved in a minimal amount of ACN and subsequently made up in 50 mM NEM to the final concentration of 600 µM. The pH was adjusted to 8.0 with 1 M HCl. A pre-reacted cysteine-bromobimane adduct was also added to the bromobimane stock that resulted in 200 pmol cysteine-bromobimane on column with every 50 µL injection. Cysteine had a precision of 7% in quality control standards and 15% in HepG2 cell samples.

On the day cells were harvested, the HepG2 cell suspension (100 µL) was used to prepare the sample for GSH analysis. HepG2 cells were centrifuged at 600 x g for 10 minutes at 4 °C to pellet the cells. The medium was removed and cells were resuspended in 50 mM NEM, pH 8 (100 µL). The bromobimane stock with cysteine internal standard (10 µL) was added to the sample. The sample was left for 1 hour in the dark at room temperature to ensure all GSH reacted with bromobimane. At the end the incubation, 100% TCA (10 µL) was added to the sample to lyse HepG2 cells and precipitate proteins. These samples were then stored in -80 °C until analysis.

All GSH standards were diluted in 50 mM NEM, pH 8. Standards had the same method preparation and stored as HepG2 cell samples. Glutathione standards included those used to make the standard curve and quality control standards. Quality control standards included 50 pmol, 100 pmol and 400 pmol on column.

At the time of analysis, samples and standards were thawed at room temperature and then centrifuged at 13,000 x g for 5 minutes. An aliquot (50 µL) of the supernatant was injected onto a 4.6 mm x 15 cm Phenomenex Prodigy 5 ODS2 column. Mobile phases consisted of solution A (0.25% acetic acid:9% ACN, pH 3.7) and solution B (75% v/v ACN). The cysteine and GSH were eluted with a mobile phase that comprised 100% A for 7 minutes followed by 100% B for 4 minutes. Each elution was then re-equilibrated with 100% A for 5 minutes. The flow rate was 1 mL/min. The fluorescence detector was set at Ex\textsubscript{394} nm:Em\textsubscript{480} nm. Typical chromatograms of a GSH standard sample and a HepG2 cell sample are shown in Figure 2.1 and Figure 2.2 respectively.
Figure 2-6  Typical chromatogram of a GSH standard (200 pmol on column) with the cysteine internal standard (200 pmol on column). The chromatogram shows good separation of cysteine and GSH. The third peak is a reagent hydrolysis peak.
Figure 2-7 Typical chromatogram of HepG2 cell sample (100 μL) with the added cysteine internal standard (200 pmol). This chromatograph was taken from the day 15 control sample. The chromatogram shows good separation of cysteine and endogenous GSH. The third peak is a reagent hydrolysis peak.

A standard curve was constructed between 0 and 600 pmol on column, as shown in Figure 2.3. The peak area of GSH was compared to the peak area of cysteine to create a ratio which was plotted against the corresponding known amount of GSH. Six points were used to make this standard curve, adhering to the FDA and EMA guidelines. The experiment was performed in quintuplicate. This range showed a linear relationship. Following the FDA and EMA guidelines, the calibration standards had ± 15% deviation from nominal concentration and lower limit of quantification standards had ± 20% deviation from nominal concentration.
2.7. Statistical methods

2.7.1. t-test
P-values less than 0.05 are considered to be statistically significant. Results in this project were analysed using an Unpaired Samples t-Test. Microsoft Excel was used to determine the p-value for t-tests.
3. RESULTS

3.1. Optimisation of literature methods

It was necessary to perform a few alterations to the published protocols (126 – 128), either to get the assay to work with this project (e.g. number of cells required), or to strengthen the confidence in the assay (e.g. addition of an internal standard), or achieve general improvement (e.g. reduction of waste). This section focuses on how these alterations not only worked but improved assays. One of the most important alterations was the addition of internal standards to the GSH and AMP/ADP/ATP HPLC assays. An internal standard allows for the correction of random or systematic errors.

3.1.1. Cell lysis preparation for the mitochondrial DNA assay

The cell number and volume of lysis buffer needed to be optimised to ensure there was enough lysis buffer to release the DNA and be at the correct concentration for a qPCR reaction. This method was optimised by PCR. The protocol for the lysis buffer was optimised by the Cree Laboratory, Department of Obstetrics and Gynaecology at the University of Auckland. The lysis buffer consisted of 0.5 ng/mL proteinase K, 0.5% Tween® 20, and 50 mM Tris-HCl, pH 8.5. Since proteinase K was the limiting factor, different concentrations (1, 2, and 5 ng/mL) were also tested to find an ideal amount. 50 µL of lysis buffer was added for every 100,000 cells. Samples were incubated at 55 °C for 16 hours and then the temperature was increased to 95 °C for 10 minutes. HepG2 cells were centrifuged for a few seconds to pellet the cellular debris and 2 µL of the supernatant was taken for PCR analysis. The DNA sample was then added to a master mix, which consisted of 1 µL autoclaved water, 1 µL forward short primer, 1 µL reverse short primer, and 5 µL Taq polymerase. The PCR protocol for a cycle was the following: denaturing at 94 °C for 45 seconds, annealing at 45 °C for 55 seconds, and extending at 72 °C for 1 minute and 30 seconds. The PCR ran for 30 cycles with a final incubation at 72°C to finish the extension. PCR products were then held at 4 °C. 0.5 ng/mL produced a strong band and thus a higher concentration of proteinase K was not required (Figure 3.1).
Figure 3-1 The effect of proteinase K concentration on the extraction of DNA from HepG2 cells. A 2% agarose gel was stained with ethidium bromide to reveal DNA products of human genes GAPDH and ND4 produced by PCR. Lane 1 includes a 1 Kb DNA ladder (Invitrogen) and lane 2 had no PCR product added. 0.5, 1, 2, and 5 correspond to the proteinase K concentration (ng/mL). Lanes 3 and 9 were the positive control, which had human DNA template that was prepared by Mike Goldthorpe, Department of Molecular Medicine and Pathology, University of Auckland. Lanes 4 and 10 were the negative control, which had sterile distilled water instead of a DNA template.
3.1.2. HPLC assay to determine AMP/ADP/ATP concentration in HepG2 cells

The original protocol also detected creatine, which was adapted as an internal standard since it is not found in cell culture. The creatine internal standard was then verified by looking at the peak creatine area data in quality control samples and all HepG2 cell samples. Quality control samples and HepG2 cell samples both had a precision of 11%. There was no significant difference between the creatine peak area of quality control samples and HepG2 cell samples.

The original protocol by Manfredi et al. stated cell samples should be centrifuged at 17,000 \( \times \) g for 10 minutes at 4 °C, the medium removed, and 0.4 M perchloric acid (100 µL) added to allow for the cell lysis (127). This was followed by 4 M potassium carbonate (10 µL) for basification (127). However, with the addition of the creatine internal standard, an adjustment to 0.4 M perchloric acid (90 µL) for cell lysis was required to allow for the addition of 20 mM creatine in 0.4 M perchloric acid (10µL). The potassium carbonate step was unchanged. To determine the baseline level of AMP/ADP/ATP in HepG2 cells, a range of 50,000 – 1,000,000 HepG2 cells were analysed with the 100 µL lysis protocol and 50 µL injection onto column. However, even with 1,000,000 cells, the ATP level was too low. AMP and ADP were difficult to distinguish against the background. Since half the prepared sample was wasted, the injection volume was increased to 100 µL. This risked disruption of the pellet, thus an alteration to the volume in preparation steps was required to ensure the pellet was not disturbed, which is stated in 2.5. These two protocols are compared in Figure 3.2.
Figure 3-2 The comparison between the original protocol (127) (red) and the altered protocol (blue). This protocol was used to prepare samples for the AMP/ADP/ATP HPLC analysis. Steps that remained unchanged are shown in yellow.
The 100 μL injection with 150 μL preparation had a greater signal for AMP, ADP, and ATP compared to the original 50 μL injection with 100 μL preparation (Figure 3.3). There is a limit to the number of HepG2 cells that can be grown in a 25 cm² flask. If more than 1,000,000 HepG2 cells were used for the AMP/ADP/ATP analysis, it would have required switching to the next largest cell culture flask. That was not ideal because it would have required more reagents and space. When HepG2 cells are at 90% confluence and split following the protocol stated in section 2.2, 1,000,000 cells is about 1 mL of the cell sample. This was determined by calculating the cells/mL using a haemocytometer and trypan blue.

![Graphs showing the effects of HepG2 cell volume on AMP, ADP, and ATP levels.](image)

Figure 3-3 The effects of the volume preparation of HepG2 cells to AMP, ADP, and ATP levels. Both 50 μL (blue) and 100 μL (red) injection started with 1 million HepG2 cells and were prepared using the protocol stated in Figure 3.2. Values shown are the mean ± SD (N = 3).
### 3.1.3. HPLC assay to determine reduced glutathione concentration in HepG2 cells

The GSH standard curve was linear up to 600 pmol on column, as shown in Figure 3.4. Amounts greater than 600 pmol on column resulted in a plateau effect.

![Figure 3-4 The preliminary GSH standard curve to determine the linear range](image)

GSH was diluted in PBS, pH 7.4 and reacted with 3 mM bromobimane in 50 mM NEM, pH 8. This was before the addition of the internal standard to this protocol. \( N = 1 \)

50 µL was injected onto the column to produce 600 pmol on column. However, standards were produced to have a volume of 100 µL resulting in 1200 pmol of GSH in the solution. Originally, 3 mM bromobimane (10 µL) was added, resulting in 30 nmol in the sample. This results in a 25-fold bromobimane excess.

The bromobimane concentration was adjusted to balance reduction in wastage whilst ensuring all GSH was reacted. The concentration of the bromobimane stock was reduced fivefold to 600 µM, resulting in 6 nmol in the sample. Since there was less bromobimane in the sample, the incubation time needed to be increased. The 600 µM bromobimane stock was tested with different incubation times and compared against the original protocol of 3 mM bromobimane with five-minute incubation (Figure 3.5). The 600 µM bromobimane stock with a 45 minute incubation resulted in a stronger GSH signal compared to the 3 mM bromobimane stock with five-minute incubation. However, the 60 minute incubation time was selected to have confidence that all GSH reacted with the bromobimane.
The second improvement to this assay was the addition of the internal standard, cysteine. Cysteine was diluted in PBS, pH 7.4. Cysteine was unstable after one freeze-thaw cycle, requiring the cysteine stock be made fresh each HPLC run. This process would have introduced variability with the weighting and diluting of cysteine. This is not ideal for an internal standard, which should be from the same stock. To avoid this problem, cysteine was pre-reacted with bromobimane to produce a stable adduct. The cysteine-bromobimane adduct was created by taking 444 μM cysteine in PBS, pH 7.4 and reacting it with 1.5 mM bromobimane. The sample was left in the dark for one hour at room temperature to ensure all cysteine had reacted. It was then diluted 10-fold into the 600 μM bromobimane stock, producing a cysteine concentration of 44.4 μM. It was added to the bromobimane stock to allow for a one-step addition. It was further diluted to 4 μM (10 μL of the stock into 100 μL of sample and 1 μL 100% TCA). With the 50 μL injection, this resulted in 200 pmol cysteine on column. A freeze-thaw experiment was performed for verification of the stability of the cysteine-bromobimane adduct and the ability of bromobimane to react with GSH. The same stock was aliquoted and then freeze thawed between one to nine times. This experiment followed the same protocol stated in METHODS 2.6 with the exception that GSH was diluted into PBS, pH 7.4. There was no change in the peak area produced by 200 pmol cysteine on column and 600 pmol GSH on column (Figure 3.6).
Figure 3-6 Effect of freeze-thawing on the stability of bromobimane-cysteine internal standard. The bromobimane-cysteine (blue line) represents 200 pmol on column. The GSH standard (red line) in PBS represents 500 pmol on column (N = 1).

When trying to determine the number of HepG2 cells required for the GSH assay, endogenous cysteine interfered with the cysteine internal standard. This is shown in Figure 3.7. The 200 pmol cysteine internal standard should result in a cysteine peak area around 1000 LU. HepG2 cells in PBS showed an increased cysteine area with an increased number of cells.

50 nM NEM, pH 8 replaced PBS, pH 7.4 in the preparation of the GSH standards and HepG2 cell samples. The change in buffer and pH prevented the reaction between endogenous cysteine and bromobimane. However, it still permitted the reaction between GSH and bromobimane. The pre-reacted cysteine in the bromobimane stock was unchanged with samples under 100,000 cells, shown in Figure 3.7.

Figure 3-7 The effects of buffer on the cysteine in HepG2 cells. The number of HepG2 cells ranged from 25,000 to 200,000. The cells were either resuspended in PBS (blue line), pH 7.4 or 50 mM NEM, pH 8 (red line). 200 pmol cysteine was added to all samples as an internal standard, which results in an area around 1000 LU. (N = 2).
The assay showed GSH saturation when using more than 100,000 cells and thus the number selected for analysis (Figure 3.8). When HepG2 cells are at 90% confluence and split following the protocol stated in section 2.6, 100,000 cells is about 100 μL of the cell sample. This was determined by calculating the cells/mL using a haemocytometer and trypan blue.

![Graph showing the effect of cell number on GSH determination.](image)

**Figure 3-8 The effect of cell number on GSH determination.** The amount of HepG2 cells ranged from 25,000 to 200,000. Values shown are mean ± SD (N = 2).

The cysteine internal standard was then verified by looking at the peak cysteine area data in quality control samples and all HepG2 cell samples. There was no significant difference between the two groups. The quality control samples had a precision of 7% and the cell samples had a precision of 15%. There was no significant difference in peak cysteine area between quality control samples and HepG2 cell samples.

### 3.2. The effects of drug concentration and exposure time on cell number

The neutral red assay was used to confirm the live HepG2 cell number was not altered between treatments and days. There were two important reasons for doing this analysis. The first was to confirm that drug treatments were not killing the HepG2 cells. The interest in these drugs and
potential combinations is potential cell damage, not cell death. The second purpose was to ensure the number of cells used for analysis was the same between treatment groups. That ensures that if a change in one or more of the endpoints occurred in a treatment group, it would be the result of intracellular response to treatment and not because of a different number of cells being analysed. This analysis was done in both the 20-day exposure to drugs at twice the maximal plasma concentration treatments and short-term exposure to drug at tenfold the maximal plasma concentration treatments.

There was no significant change between the treatment groups and control, indicating that the drugs at ten-fold the maximal plasma concentration treatment exposure for 2.5 hours did not result in noticeable cell death (Figure 3.9).

**Figure 3-9** The effect of short-term exposure of HepG2 cells to drugs at ten-fold maximal plasma concentration on cell number. Cell number was indirectly determined by neutral red, which can only be absorbed by viable cells. The cellular neutral red level was determined by the absorbance at 540 nm. Values shown are the mean ± SD (N = 2).
There was no significant change between the treatment groups compared to the control on the same day (Figure 3.10). There was also no significant difference between the treatment groups on different days. This proves the live cell numbers were relatively the same between all samples and days.

Figure 3-10 The effect of twenty day exposure of HepG2 cells to drugs at twice maximal plasma concentrations on cell number. Cell number was indirectly determined by neutral red, which can only be absorbed by viable cells. The cellular neutral red level was determined by the absorbance at 540 nm. Values shown are the mean ± SD (N = 2).
3.3. The effect of short-term exposure of HepG2 cells to drugs at tenfold maximal drug plasma concentration

3.3.1. The effect on mitochondrial DNA level

The short-term exposure to drugs at tenfold the maximal plasma concentration had no significant effect on the mtDNA levels in HepG2 cells for any combination (Figure 3.11).

![Graph showing the effect of short-term exposure of HepG2 cells to drugs at tenfold maximal plasma concentration on mtDNA level.](image)

Figure 3-11 The effect of short-term exposure of HepG2 cells to drugs at ten-fold drugs at maximal plasma concentration on mtDNA level. mtDNA levels were determined by measuring ND4, a gene on the mitochondrial genome. This was then taken as a ratio to the measurement of GAPDH, a gene on the nuclear genome. Columns shown the mean ± SD (N = 3).

3.3.2. The effect on AMP/ADP/ATP level

The cyclosporine/telbivudine combination was the only treatment group that had a significant difference from the control (Figure 3.12). It had a significant increase in both the amount of AMP and ATP (p values 0.0365 and 0.0124 respectively). There were no other significant changes in AMP, ADP, and ATP in the other treatment groups.
Figure 3-12 The effect of short-term exposure of HepG2 cells to drugs at ten-fold maximal plasma concentration on AMP (A), ADP (B), and ATP (C) levels. Columns shown the mean ± SD (N = 3). *CT had a significant increase in AMP compared to the control (p value = 0.0365). **CT had a significant (p = 0.0124) increase in ATP compared to the control.
3.3.3. The effect on reduced glutathione level

Only three treatments groups produced a significant change in the amount of GSH in cells. The cyclosporine/telbivudine combination had a significant increase in GSH (p value = 0.017). The cyclosporine/didanosine/lamivudine combination also had a significant increase in GSH (p value = 0.002). The lamivudine/telbivudine combination was the only treatment to have a significant decrease in GSH (p value = 0.016). These are shown in Figure 3.13. All other treatment groups showed no significant change.

Figure 3-13 The effect of short-term exposure of HepG2 cells to drugs at ten-fold maximal plasma concentration on GSH level. Columns shown the mean ± SD (N = 3). *CT had a significant GSH increase compared to the control (p value = 0.017). **LT had a significant GSH decrease compared to the control (p value = 0.016). ***CDL had a significant GSH increase compared to the control (p value = 0.002).
3.4. The effect of 20-day exposure of HepG2 cells to drugs at twice maximal drug plasma concentration

3.4.1. The effect on mitochondrial DNA level

The majority of the treatment groups had a decrease in mtDNA levels compared to the control. However, on certain days a few treatments did have higher mtDNA levels compared to the control, as shown in Figure 3.14. This increase in mtDNA was not consistent between the three days of mtDNA analysis. The results also have large error bars. Unlike, the long term GSH and ATP results, mtDNA did not show a trend in all samples between day 10 and day 20. The mtDNA levels in the control sample did not change.

![Figure 3.14](image)

Figure 3-14 The effect of long-term exposure of HepG2 cells to drugs at twice the maximal drug plasma concentration on mtDNA level. The mtDNA levels were determined by measuring ND4, a gene on the human mitochondrial genome. This was then taken as a ratio to the measurement of GAPDH, a gene on the human nuclear genome. Columns shown the mean ± SD (N = 3).
All treatment groups except the combination of didanosine/lamivudine had at least one day with a significant difference in mtDNA level compared to the control. These treatment groups are compared individually against the control in Figure 3.15 with the days of significant difference noted by the asterisk. All treatment groups with a significant difference from the control had a reduction in mtDNA level. The addition of more drugs to the combination did not result in greater mtDNA reduction. However, the combination of cyclosporine/didanosine/lamivudine did result in the greatest reduction in mtDNA levels (Figure 3.15 – K).
Figure 3-15 The effect of long-term exposure of HepG2 cells to drugs at twice the maximal plasma concentration on mtDNA level (separated). The mtDNA levels were determined by measuring ND4, a gene on the human mitochondrial genome. This was then taken as a ratio to the measurement of GAPDH, a gene on the human nuclear genome. Columns shown the mean ± SD (N = 3). *Days when the mtDNA levels in the treatment groups had a significant difference (p value less than 0.05) from the mtDNA level in the control.
3.4.2. The effect on AMP/ADP/ATP level
The majority of samples did not show a significant difference in AMP levels from day 10 to day 20 (Figure 3.16). The control, the didanosine single treatment, lamivudine single treatment and cyclosporine/didanosine combination had a significant decrease in AMP levels from day 10 to day 20. The telbivudine treatment had a significant increase in AMP levels from day 10 to day 20.

Figure 3-16 The effect of long-term exposure of HepG2 cells to drugs at twice the maximal plasma concentration on AMP level. Values shown are mean ± SD (N = 3).

The cyclosporine single treatment, telbivudine single treatment, cyclosporine/telbivudine combination, didanosine/lamivudine combination, didanosine/telbivudine combination, and cyclosporine/didanosine/lamivudine combination had a significant decrease in AMP compared to the control (Figure 3.17). This only occurred in day 10. No other days in this 20-day exposure to drugs at twice the maximal plasma concentration treatments showed a significant change in AMP levels compared to the control.
Figure 3-17 The effect of long-term exposure of HepG2 cells to drugs at twice the maximal plasma concentration on AMP level (separated). Values shown are mean ± SD (N = 3). *Days when the AMP levels in treatment groups had a significant difference (p value less than 0.05) from the AMP level in the control.
The cyclosporine treatment, telbivudine treatment, didanosine/telbivudine combination, cyclosporine/didanosine/telbivudine combination and cyclosporine/didanosine/lamivudine/telbivudine combination all had a significant increase in ADP levels. This is shown in Figure 3.18. All other samples did not show a significant difference between day 10 and day 20.

![Graph showing the effect of long-term exposure of HepG2 cells to drugs at twice the maximal plasma concentration on ADP level. Values shown are mean ± SD (N = 3).](image-url)

Figure 3-18 The effect of long-term exposure of HepG2 cells to drugs at twice the maximal plasma concentration on ADP level. Values shown are mean ± SD (N = 3).
The didanosine/telbivudine combination, cyclosporine/didanosine/telbivudine combination, and cyclosporine/didanosine/ lamivudine/telbivudine combination had a significant decrease in the ADP levels compared to the control in day 10. This is shown in Figure 3.19. No other days in the treatment combinations had a highly significant decrease in ADP compared to the control.

Figure 3-19 The effect of long-term exposure of HepG2 cells to drugs at twice the maximal plasma concentration on ADP level (separated). Values shown are mean ± SD (N = 3). *Days when ADP levels in the treatment had a significant difference (p value less than 0.05) from the ADP levels in the control.
There is a noticeable upward trend in the ATP levels from day 10 to day 20 (Figure 3.20). The samples that did not have a significant change in ATP levels from day 10 to 20 include cyclosporine/didanosine combination, cyclosporine/telbivudine combination, and cyclosporine/didanosine/lamivudine/telbivudine combination. All other treatments had a significant increase in ATP levels from day 10 to day 20.

Figure 3-20 The effect of long-term exposure of HepG2 cells to drugs at twice the maximal plasma concentration on ATP level. Values shown are mean ± SD (N = 3).
Lamivudine treatment and telbivudine treatment are the only two groups that showed a significant change in ATP levels compared to the control (Figure 3.21). Lamivudine treatment had a higher ATP level compared to the control on all days, but only had a statically significant difference from the control on day 20. Telbivudine had a significant difference from the control on day 10 and day 20, with a lower and higher ATP levels, respectively. All other treatment combinations showed no significant difference from the control.

Figure 3-21 The effect of long-term exposure of HepG2 cells to drugs at twice the maximal plasma concentration on ATP level (separated). Values shown are mean ± SD (N = 3). *Days when the ATP levels in the treatment groups had a significant difference (p value less than 0.05) from the ATP level in the control.
3.4.3. The effect on reduced glutathione level

There is a noticeable downward trend in GSH levels in the majority of the samples. 13/16 treatment groups had a significant decrease in GSH from day 10 to day 20 (P value less than 0.05). This is shown in Figure 3.22. The only three treatments did not result in a significant change in GSH levels from day 10 to day 20. These were the telbivudine treatment, cyclosporine/didanosine combination and cyclosporine/telbivudine combination.

![Graph showing the effect of long-term exposure of HepG2 cells to drugs at twice the maximal plasma concentration on GSH level. Values shown are the mean ± SD (N = 3).](image)

Figure 3-22 The effect of long-term exposure of HepG2 cells to drugs at twice the maximal plasma concentration on GSH level. Values shown are the mean ± SD (N = 3).

The treatment combinations that resulted in a significant GSH difference (p value less than 0.05) from the control were didanosine single treatment, telbivudine single treatment, cyclosporine/lamivudine combination, cyclosporine/telbivudine combination, didanosine/lamivudine combination, cyclosporine/didanosine/lamivudine combination and cyclosporine/didanosine/lamivudine/telbivudine combination. All other treatment combinations did not have a significant GSH level change from the control. The majority of the significant changes had an increase in GSH. The exceptions are
didanosine single treatment at day 15 and cyclosporine/telbivudine combination at day 15, which had a significant decrease in GSH. This is shown in Figure 3.23. None of the treatment combinations had a significant difference in all three days.

Figure 3.23  The effect of long-term exposure of HepG2 cells to drugs at twice the maximal plasma concentration on GSH (separated). Values shown are mean ± SD (N = 3). *Days when the GSH level in the drug treatment had a significant difference (p value < 0.05) from the GSH level in the control.
4. DISCUSSION

4.1. Background
Adverse drug reactions (ADRs) commonly manifest in the liver because it is the primary site for drug metabolism (8). ADRs are broadly classified into two groups: predictable and dose-dependent or idiosyncratic (4). It is unlikely that idiosyncratic drug induced liver injury (DILI) will be observed in clinical trials because its incidence is too low (9, 11). Even after a drug has been approved for market, DILI occurrence is underestimated due to lack of or incomplete reporting, or misdiagnosis (9). A recently suggested mechanism for idiosyncratic DILI has been identified as drug-induced mitochondrial toxicity (18). Nucleoside reverse transcriptase inhibitors (NRTIs) are known mitochondrial toxins due to their ability to inhibit polymerase-γ (35). Cyclosporine is known to produce reactive oxygen species (ROS) by suggested mechanisms such as increase mitochondrial Ca$^{2+}$ concentration, blockage of the permeability transition pore, and alteration to the electron transport chain (ETC) (112). Since NRTIs and cyclosporine are suggested to cause mitochondrial toxicity, they were selected for this project.

4.2. Experimental design
Preclinical experiments are limited in their ability to detect idiosyncratic toxicity because there is no ‘one size fit all’ method (1). Regulatory animal testing and in vitro experiments are used to predict potential human drug toxicity (129). However, there is no animal that is ideal to study DILI (15). This is believed to be a result of several factors. First, the metabolism in animals does not reflect the metabolism in humans (129). Animal testing is a controlled environment and does not reflect the diversity of humans or their heterogeneous conditions (129). The animals used are “healthy,” and often genetically engineered and/or inbred (1,129). Since preclinical animal testing is extremely poor in predicting human hepatotoxicity (12), it was not selected for this project.

An animal study was also a poor choice because it provides information about drug effects in a whole organism (12). This project was focused on understanding the mechanism of DILI. An in vitro study allowed for the possibility for a more controlled and simplistic system (130). This also provided a chance to study the effects in human cells (12).
Two potential *in vitro* methods were considered for this project: primary human hepatocytes and a hepatocyte cell line. Hepatocytes were selected because they are the predominant cell type in livers (120). Fresh human hepatocytes are a valuable screening tool (8) and are the gold standard for *in vitro* testing because they better reflect the *in vivo* environment (131). Primary hepatocytes are the preferred *in vitro* system to study mechanistic toxicity since they retain more key hepatic functions (12). For example, they are a more variable population due to inter-individual differences of donors (131). However, metabolic ability was not considered a primary importance in this project because the NRTIs are eliminated unchanged (35). Cyclosporine is the only drug of interest that is metabolised (113). Primary cells hepatocytes have an unstable phenotype that can vary depending on the patient’s status at the time of collection (132,133).

Primary hepatocytes are limited by availability and short life (8), which is influenced by culture conditions (12). Primary hepatocytes have a functional period between 24-72 hours (134). However, cell function will dramatically decrease with time (134). Once again, this was not ideal for this study because half of this project investigated longer-term toxicity. In contrast, hepatic cell lines have benefits of being easily available, and having stable phenotype and unlimited life span (135). This project had 16 potential drug combinations. The cells needed to have a consistent phenotype, robustness, and reproducibility, which is why a hepatocyte cell line was selected over primary hepatocyte cultures (8). For those reasons, a hepatocyte cell line was a better candidate than primary hepatocytes for this project.

HepG2 cells were selected for this project because they have a high content of mitochondria, making it an excellent cell line to study mitochondrial toxicity (132). They are also the most widely-used human hepatic cell line (135).

The protocols selected to analyse cell viability, GSH, and ATP levels were carefully considered since there are multiple methods available. Originally cell number was determined using trypan blue. However, this was time-consuming and inaccurate with the large number of samples that needed to be analysed. However, it did provide a rough estimate of cell suspension volume required to get a desired number of cells. The trypan blue analysis for cell viability was replaced with neutral red. Neutral red is an amphiphilic cationic dye that is absorbed by lysosomes and binds to the lysosomal matrix of viable cells (33). Neutral red enters cytoplasm in non-ionic lipophilic form (136). At
physiological pH, neutral red has a no charge (34). This allows it to enter the cellular membrane (34). When it enters lysosomes, the low pH protonates the dye to the cationic form and traps it (136). The amount of neutral red that becomes trapped in the lysosome depends on the pH of the lysosome, the lysosomal membrane proton pump efficiency, and the condition of the lysosomal membrane (136), thus it can only accumulate in lysosomes of viable, undamaged cells (137). After incubation with neutral red, it is extracted from viable cells using an acidified ethanol solution (34). The dye is then quantified by a spectrophotometer at 540 nm, which indirectly measures the number of viable cells (34). Neutral red can leak out of the lysosome if there is damage to the lysosomal membrane or inhibition of the proton pump (136), thus dead cells or cell with damaged lysosomes will not maintain the dye (34). Neutral red is also quantifiable, inexpensive, stable, and simple to use (34). Neutral red has a long experimental usage, thus proving the strength and practicality of this assay (138).

The Manfredi et al HPLC protocol was selected to analyse ATP levels because it also allowed for simultaneous analysis of AMP and ADP (127). Measuring AMP and ADP is important because they provide a confirmation of an ATP reduction since it will either become ADP or AMP. It is also important to understand if ATP is becoming AMP or ADP because it provides insight into severity of damage. When mitochondria are unable to sustain ATP demands, cells will convert two ADP to one ATP and one AMP via adenylate kinase (50). AMP will go on to interact with AMP-activated protein kinase, which will in turn off ATP-consuming pathways (50). This protocol was also selected because it used a reverse phase technique with an ion-paired reagent to neutralise the charge on nucleotide phosphates (127). Not only does reverse-phase chromatography allow for shorter separation times, but also it has better reproducibility (127).

GSH can be difficult to measure accurately because of spontaneous oxidation of GSH in the presence of molecular oxygen (65). Oxidation of GSH is dependent on temperature and pH, both of which are suggested to be kept low to prevent oxidation (65). N-Ethylmaleimide, an alkylating agent, is also suggested as a buffer to prevent thiol oxidation (65). The method that was selected for this project was developed by Cotgreave and Moldeus (128). It was selected because it is an HPLC method with fluorescence detection, which is extremely sensitive and selective (65,128). The Cotgreave and Moldeus method has the ability to measure all thiol-containing components (128).
Another problem with analysing intracellular GSH cell lysis results in either rapid oxidation to GSSG, degradation by extracellular γ-glutamyl transpeptidase, or creation of artefacts (128,139). Cotgreave and Mouldeus overcame these problems by selecting bromobimane, which reacts with thiol groups to produce fluorescence adduct and can pass freely through a cell membrane without damaging it (128). The HPLC analysis has good chromatographic separation and fluorescence is an ideal detection method because it has excellent sensitivity (128).

4.3. Effects of drug exposure time, concentration, and combination on mitochondrial endpoints in HepG2 cells

As mentioned in section 4.1, there are four drugs of interest in this project: cyclosporine, didanosine, lamivudine, and telbivudine. Venhoff et al performed a similar study with HIV NRTIs in HepG2 cells (84). The authors were interested in combination toxicity because even though clinically NRTIs are used in combination to treat HIV, a majority of safety data only examines toxicity of monotherapy (84). Their study showed a large majority of NRTI combinations resulted in an additive or synergistic toxicity (84). However, a review of the literature could not find a similar study regarding NRTIs used to treat CHB. This project not only addressed this void, but also other additional treatments due to common co-morbidities.

The first goal of this project was to determine if there was an immediate mitochondrial toxicity from these selected drugs, either alone or in combination. A concentration tenfold the maximal drug plasma concentration was selected in the hope it would produce a more dramatic response. This drug concentration did not result in massive cell death (Figure 3.9). Changes to mitochondrial endpoints did not occur in the majority of samples (Figures 3.11 – 13). It is expected that treatment would not alter mtDNA levels because NRTI toxicity requires an accumulation of mtDNA defects, which occurs after prolonged use (13). Cyclosporine/telbivudine was the only combination that produced a significant change in AMP level and ATP level compared to the control (Figure 3.12). An increase in both AMP and ATP could be the result of two ADPs being converted to AMP and ATP, catalysed by acetylate kinase (50). This occurs when mitochondria are unable to meet ATP demands (50). However, the ADP level was not significantly different from the control in the cyclosporine/telbivudine treatment.
The GSH analysis provided more insight into potential effects of these drugs in combination. Three treatment groups had a significant change in GSH level compared to the control (Figure 3.13). The cyclosporine/didanosine/lamivudine combination resulted in a significant increase in GSH level. However, a review of the literature not only failed to provide an explanation for this result because the combination was never studied before.

The cyclosporine/telbivudine combination resulted in a significant increase in GSH levels. There was only one article that examined cyclosporine and telbivudine in combination. Zhou et al treated 20 healthy male patients with 600 mg/day telbivudine and 4 mg/kg/day cyclosporine (124). On the first day, the patients were split into two groups and started either telbivudine or cyclosporine treatment (124). On the fifth day, the other drug was added to the regimen (124). Treatment ended on the ninth day (124). The design of this trial does not reflect the clinical setting; diseased patients were not selected for this trial and both drugs are used long-term (35, 112). The authors observed no changes in pharmacokinetic properties of either drug compared to when they were used alone (124). This article provided little clarification for the result in this project as the authors only examined pharmacokinetic interactions (124); there were no observations for ADRs and clinical effectiveness could not be monitored since they did not select diseased patients (124).

The lamivudine/telbivudine combination produced a significant decrease in GSH levels. This immediate reduction in GSH following combination exposure is a concern because it will reduce the cellular buffer capacity for oxidative stress (66). Lamivudine and telbivudine have been studied more extensively in combination due to the interest of potentially using it as a bitherapy to treat CHB. Zhou et al performed a phase I clinical trial examining pharmacokinetics of lamivudine and telbivudine in combination with sixteen healthy male volunteers divided into two groups (140). The first group was administered 200 mg/day of telbivudine from days 1 – 7 and from day 8 – 14, the subjects added 100 mg/day of lamivudine (140). The second group had the same dosage, but they started with lamivudine and then added telbivudine (140). The authors found no change in pharmacokinetic properties when the drugs were coadministered (140). However, the dose for telbivudine was lower than the current clinical dosage (600 mg/day) (2). This brings into question the relevance of their results since clinically the dose for telbivudine is three-fold higher than what they tested. This is extremely important in NRTI experiments because its toxicity is dose dependent (37). These results
also have limited clinical application because the authors used healthy patients and the short length of the trial.

Lai et al addressed these issues that limited the results in the Zhou et al article (104). The authors performed a one year trial studying the efficacy and safety of telbivudine alone, lamivudine alone, and then both used in combination (104). Telbivudine dosage included 400 mg/day and 600 mg/day (104). Lamivudine was used at 100 mg/day (104). 104 patients were in this study and their treatment was evenly randomised between the five potential groups (104). HBV DNA levels were reduced within the first six months across all groups and treatments were well-tolerated (104). CHB patients had a significantly better virologic response on telbivudine compared to lamivudine (104). The combination treatment also had a similar response to telbivudine alone; suggesting the addition of lamivudine has no increased benefit to treatment (104). The GSH depletion in the lamivudine/telbivudine combination could explain the lack of improvement when lamivudine was added to telbivudine therapy. However, there is no information in the literature regarding the effects of lamivudine and telbivudine on GSH level, so this could not be confirmed. The study also showed no pharmacokinetic interaction between lamivudine and telbivudine (104).

However, there is evidence of potential cross-resistance between lamivudine and telbivudine (81). Telbivudine is not recommended if lamivudine failure has occurred and vice versa (81); therefore it is unlikely that lamivudine and telbivudine would be used in combination due to similar mechanism of action and drug-resistance (141).

Even though these highlighted combinations resulted in a significant change in a mitochondrial endpoint from the control, there was no trend in damage accumulation with the addition of drugs to the combination. There is confidence that these drugs do not cause immediate mitochondrial damage because a high drug concentration was tested and only had a few combinations that had a significant change in the mitochondrial endpoints, which are unlikely to have an effect in an in vivo setting.

The long-term survival of HIV-positive patients has revealed the potential chronic toxicity of antiretroviral treatment (82). Long-term toxicity has also been seen with cyclosporine with a majority of patients experiencing abnormal renal function (113). For that reason, it was important to study long-term toxicity of these drug combinations.
This was performed in HepG2 cells with a drug concentration of twice the maximal drug plasma concentration over twenty days. A continuation of the tenfold maximal drug plasma concentration did not occur because it was proven to be too toxic in HepG2 cells (84). Twice the maximal drug plasma concentration is a more clinically-relevant concentration.

Cell viability showed no changes between treatment compared to the control on the day and no difference within treatment groups on different days (Figure 3.10). Treatment had no effect on cell growth, nor did it result in cell death. It is also important because any observed changes in a mitochondrial endpoint are a result of a treatment and not due to different cell number.

The 20-day drug exposure had the most dramatic effect on mtDNA level. All but one treatment resulted in a significant reduction in mtDNA level compared to the control (Figures 3.14 and 3.15). This outcome is not surprising because NRTIs are known inhibitors of polymerase-γ (35). Cyclosporine could also indirectly damage mtDNA due to its ability to induce ROS production (12, 112).

The only treatment that did not result in a significant change in the mtDNA level was the didanosine/lamivudine combination. A similar result was seen in Venhoff et al, who were also studying NRTI combination toxicity in HepG2 cells (84). The authors found that the addition of lamivudine reduced didanosine toxicity in both the C_{max} and 10X C_{max} concentrations (84). This suggests some protective benefit (84).

Didanosine and lamivudine are commonly used together clinically because the combination is effective in HIV treatment (80, 142). It has excellent initial tolerance, is available in a once-daily treatment, and has low mitochondrial toxicity. However, it has a greater potential to cause long-term toxicity (143). A projective benefit has not been studied clinically and its potential combination toxicity is debatable. Clinical trials and case reports vary in the safety evaluation of the didanosine/lamivudine combination.

Berenguer et al studied the difference of lamivudine/efavirenz treatment used in combination with zidovudine or didanosine (144). For 48 weeks, 186 HIV-positive patients were on the combination with didanosine and 183 on the combination with zidovudine (144). The authors found that the didanosine/lamivudine/efavirenz combination had a more rapid increase in CD4 cell count, but at 48
weeks there was no significant difference between treatment groups (144). Treatment failure was rare and there was no significant difference between groups (144).

Palacios et al examined clinical outcomes of 147 patients who switched from didanosine/lamivudine treatment to tenofovir/emtricitabine treatment (143). The majority of ADRs improved with the switch: 100% of peripheral neuropathy, 83.3% of dyslipidaemia, 84.9% of hepatic toxicity, and 71.4% of glucose metabolism disorders (143). The only ADR that did not show major improvement was lipodystrophy, which only resolved in 14.9% of cases (143). However, the authors in this study believed the toxicity was related to didanosine (143,145).

Chang et al also presented a case study of a patient on didanosine, lamivudine, and nevirapine combination treatment that experienced non-cirrhotic portal hypertension (92). The HIV-positive patient had been on didanosine, lamivudine, and nevirapine combination treatment for 34 months without a previous history of alcohol/drug abuse, liver disease, or opportunistic infections (92). The thrombosis in the portal vein was confirmed by abdominal computerized tomography and the patient was treated with spironolactone and furosemide (92). After six days in the hospital the patient was discharged before a liver biopsy was performed because he felt considerably better (92). His treatment was altered to exchange didanosine with abacavir (92). Three months later the patient died from respiratory failure and upper gastrointestinal bleeding (92). The authors theorized that the non-cirrhotic portal hypertension was only induced by the didanosine treatment (92).

It is not surprising that even though the results in this project and Venhoff et al show a protective benefit with the didanosine/lamivudine combination, it is not reflected clinically. This is often a downside of cell culture because it might be missing critical mechanisms required for cellular injury (130).

Since the mitochondrial genome encodes proteins in the ETC, the next important endpoint to examine was ATP level. Since the majority of treatments resulted in mtDNA depletion, it was predicted that there would have been a reduction of some of the components of the ETC, thus decreasing ATP production (50). However, only two treatment groups had a significant change in ATP levels compared to the control (Figure 3.21). Telbivudine had a significant decrease in ATP on day 10. However, the other results did not reflect the predicted theory. Lamivudine had an increase in ATP in day 20. Telbivudine treated HepG2 cells also appeared to recover from the ATP depletion, with the
day 15 measurement having no significant change from the control, and day 20 having a significant increase in ATP. A correlation between changes in ATP with changes in AMP or ADP levels is expected. However, neither of these treatments had a significant difference in ADP level. Telbivudine only had a significant decrease in AMP levels at day 10. Since there a lack of balanced change between AMP, ADP, and ATP levels, the significant differences in these two treatments are unlikely to have an effect in vivo.

Other treatments did produce significant changes in AMP and/or ADP levels (Figure 3.17 and Figure 3.19, respectively). However, it had a similar problem with the lack of balanced change between AMP, ADP, and ATP.

In regards to the effects of long-term treatment on GSH level, seven treatment groups displayed a significant difference in GSH compared to the control, all with an increase in GSH. The treatments included telbivudine, cyclosporine/lamivudine, didanosine/lamivudine, cyclosporine/didanosine/lamivudine, and cyclosporine/didanosine/lamivudine/telbivudine. There is no data on these treatments’ effects on GSH level. It has been suggested in this project and in a study by Venhoff et al that didanosine/lamivudine in combination might have a protective benefit. The increase in GSH level can further support this theory.

It is interesting that even though mtDNA levels decreased in the majority of treatment groups during 20-day drug exposure, there were no major downstream effects to ATP and GSH levels. Mitochondrial damage is not always reflected by mtDNA level because cellular adaptation can occur (47). This can occur with alternations to transcription, translation, or degradation of mtRNA or proteins (48,146). However, in order to prove if and how this potential adaptation occurred, this project would also need to measure mtRNA levels and mitochondrial protein activity (84, 146).

However, even though there are significant changes in these mitochondrial endpoints for both the high dose short-term data and clinically relevant dose long-term data, there is no accumulation of mitochondrial damage with the increase number of drugs used in a combination (i.e. quadruple therapy > tritherapy > bitherapy > monotherapy) . In regards to the 20-day exposure results, the majority of significant changes only occurred in one out of three observation days and generally in the first two days of observation. It is expected that if mitochondrial damage occurred, it would get increasingly worse from day 10 to 20 because mitochondrial damage is time dependent (37). Finally,
damage in mitochondria is extremely interlinked, as shown in Figure 1.1. Even if these drugs only
targeted one mitochondrial factor, it was expected to escalate and alter other endpoints.

The increase in mtDNA mutations and/or decrease in mtDNA level can result in a lower transcription
of mtRNA (147). This in turn can decrease the amount of mitochondrial encoded proteins (147). The
reduction of mitochondrial genome encoded proteins will result in the backflow of electrons and their
accumulation in complex I and III, promoting the formation of superoxide (50). This can lead to
oxidative stress and reduced energy production (12). A reduction in ATP also prevents GSH
production because it is an energy dependent reaction (62). The disbalance between ROS and
antioxidant production will compound GSH depletion (12). If GSH becomes depleted, both free
radicals and reactive metabolites will increase until it exceeds the cellular antioxidant capacity and
cause oxidative stress (12). Excess ROS can go on to damage cellular DNA, lipids, and proteins,
altering their normal function (12). These endpoints not only directly related to each other, but also will
viciously perpetuate each other.

However, this did not occur and thus it can be concluded cyclosporine, didanosine, lamivudine, and
telbivudine alone or in combination at high dose short-term or clinically relevant dose over twenty
days, do not result in mitochondrial toxicity.

4.4. Effects of 20-day HepG2 culture mitochondrial endpoints

The control sample was very important for the long-term experiment because cell lines are highly
sensitive to passage state (148). They can fluctuate and become modified with long term use (148).
The long-term culturing can result in the evolution of sub-lines (148). In this 20-day experiment, there
was a notable trend in the majority of samples, including the control, for ATP and GSH levels. Since
the control was also affected, this is cannot be a result of drug exposure. This suggests that the
problem arose from the in vitro method. The literature did not provide a clear explanation to why this
occurred and the other endpoints did not change between days.

There was a notable upward trend in ATP levels from day 10 to day 20 in all treatment samples
except three combinations (Figure 3.19). However, this increase in ATP was not paralleled by trend
changes in AMP or ADP levels. A review of the literature did not provide any explication or other
e samples.
There was a noticeable downward trend in the amount of GSH from the day 10 to the day 20 cell samples (Figure 3.13). Again, this change occurred in all but three combinations. Two of these combinations did not have a significant change in both ATP and GSH from day 10 to day 20. This was the cyclosporine/didanosine combination and the cyclosporine/telbivudine combination. Telbivudine only had only a significant change in GSH from day 10 to day 20. The cyclosporine/lamivudine/telbivudine combination only had a significant change in ATP from day 10 to day 20.

The decrease in GSH is a concern because ten out of 13 of these treatment groups had a 20% or more reduction in GSH. A 20 – 30% depletion of GSH impairs cellular detoxification defence, resulting in cell injury and potentially death (61). Although there is literature examining its effect GSH level in vitro, none of these studies use HepG2 cells or similar timeframe.

Nkabyo et al examined the progression from proliferation to contact inhibition and spontaneous differentiation in Caco-2 cells, a colon carcinoma cell line (149). During proliferation, there was an increase in GSH precursors and GSH concentration to prepare for cellular division (149). GSH levels decreased with a reduction in proliferation and differentiation (149). This article is not a perfect example to explain the results in this project because different cell types were used. When HepG2 cells were taken for analysis they were all at 90% confluence, so the difference in GSH amount seen is not a result of proliferation. It does provide an idea that with the twenty day exposure experiment, HepG2 cells could have differentiated. However, there were no markers in this project checking if these cells had differentiated, so this theory cannot be confirmed.

The results seen in the Nkabyo et al article was also seen in a study performed by Kirin et al (72). The authors also looked at a colon carcinoma cell line (72). However, they used Ht29 cells (72). The authors found a decrease in GSH when there was a reduction in proliferation (72). Ht29 cells had a doubling time of 22 hours (72). GSH concentration was stable up to 24 hours (72). However, at 48 and 72 hours there was a decrease in GSH to 57% and 48% respectively (72). It is suggested that the increase in GSH concentration during proliferation is a protective measure because active growth makes a cell more susceptible to toxins and radiation (73).

Yuan et al did examine the difference in early-passage and late-passage of three different human diploid fibroblasts (150). They selected three cell lines, TIG-3, TIG-7 and MRC-5 (150). The authors
compared the difference between these cells while they were in 20 – 30 and 60 – 70 population doubling levels (150). They found that late passage cells had increased oxidative stress and a 24-44% reduction in GSH on a per cell basis (150). Although these are different cell lines, it is a similar result to what was seen in this project. Torrao et al states that the aging process not only has a progressive decline of GSH to GSSG, but there is also a decline of cysteine to cystine (69).

The best hypothesis in regards to this project is that GSH reduction was a result of an increase in ATP production. ROS are a normal by-product of oxidative phosphorylation, which could have reduced GSH level (12). However, that is an extremely limited theory since there are no additional endpoints in this project to study how ATP production could have been up-regulated or to measure ROS level directly. Finally, there is no literature supporting or explaining these findings.

4.5. Potential improvements

The results from this project suggest that drug exposure times and concentrations do not result in mitochondrial toxicity. However, there are a few additions that could help strengthen this result. First, one major limitation in this project was the drug concentrations tested. A suggested minimum of 5 – 6 concentrations over a wide range is recommended to produce a dose-viability curve (12). However, in this study, only two concentrations were tested. The project would have also benefited from additional fold-differences from the maximal plasma concentration since NRTI induced mitochondrial toxicity is dose dependent (37). Using higher concentrations, specifically in the long-term study could have resulted in mitochondrial damage. It is likely that the concentration in the liver is higher than maximal plasma concentration since cyclosporine, didanosine, lamivudine, and telbivudine are orally administered, thus the liver will be the first site of exposure (8, 108, 151, 152). However, the maximal drug plasma concentration is commonly selected for in experiments because the concentration at the site of action is unknown. Determining the maximal drug plasma concentration is simple because requires a blood sample. However, with any large increase in concentration it still does question if it is still clinically relevant.

Another potential improvement to this project would have been an inclusion of endpoints measuring mtRNA and COXII/COXIV activity ratio. This could have been included because even if there is a reduction in mtDNA levels, transcription and translation can be altered and mitochondrial damage
might not occur (45). That is possible in this case since there was no depletion in ATP and GSH levels. However, developing an additional two assays would not have fit within the timeframe allocated to this project.

4.6. Conclusion
The results from this project showed that length of exposure and concentration of cyclosporine, didanosine, lamivudine, and telbivudine alone or in combination did not result in HepG2 cell damage. The markers used in this experiment included cell viability, and GSH, AMP/ADP/ATP, and mtDNA levels. The use of multiple and interrelated endpoints provide strength to this null result. Future experiments with more concentrations and additional mitochondrial toxicity endpoints would further support these results.
# APPENDIX

## Appendix 1: Buffers, solutions, and gels

The following are the formulae for the various buffers, solutions, and gels used in this project.

<table>
<thead>
<tr>
<th>Solution</th>
<th>Recipe</th>
</tr>
</thead>
<tbody>
<tr>
<td>MEM Alpha 10% FCS</td>
<td>foetal bovine serum (5mL) + MEM Alpha (45 mL)</td>
</tr>
<tr>
<td>Trypsin 1X</td>
<td>10X trypsin (5mL) + 1X sterile PBS (45 mL)</td>
</tr>
<tr>
<td>Deabsorb</td>
<td>acetic acid (1 mL) + 100% ethanol (50 mL) + MilliQ H₂O (50 mL)</td>
</tr>
<tr>
<td>Glutathione HPLC Assay Solution A - 0.25% acetic acid, 9% ACN (pH 3.7)</td>
<td>acetic acid (2.5 mL) + ACN (90 mL) + MilliQ H₂O (910 mL). pH was adjusted to 3.7 with 10 M NaOH. Solution was filtered.</td>
</tr>
<tr>
<td>Glutathione HPLC Assay Solution B - 75% ACN</td>
<td>ACN (750 mL) + MilliQ H₂O (250 mL). Solution was filtered.</td>
</tr>
<tr>
<td>100% TCA</td>
<td>TCA (5 g) + MilliQ H₂O (22.7 mL)</td>
</tr>
<tr>
<td>AMP/ADP/ATP HPLC Assay Solution A - 100 mg/L tetrabutylammonium, 25 mM sodium phosphate (pH 5)</td>
<td>sodium phosphate monobasic, monohydrate (3449.5 mg) + 50 mM tetrabutylammonium phosphate (60 mL) + MilliQ H₂O (940 mL). pH was adjusted to 5 with 10% acetic acid. Solution was filtered.</td>
</tr>
<tr>
<td>AMP/ADP/ATP HPLC Assay Solution B - 100 mg/ml tetrabutylammonium, 200 mM sodium phosphate (pH 4)</td>
<td>sodium phosphate monobasic, monohydrate (27.6 g) + 50 mM tetrabutylammonium (60 mL) + ACN (100 mL) + MilliQ H₂O (840 mL). pH was adjusted to 4 with 100% acetic acid. Solution was filtered.</td>
</tr>
<tr>
<td>0.4 M Perchloric acid</td>
<td>70% perchloric acid (7.1 mL) + MilliQ H₂O (240 mL)</td>
</tr>
<tr>
<td>4 M Potassium carbonate</td>
<td>potassium carbonate (5.53 g) + MilliQ H₂O (10 mL)</td>
</tr>
<tr>
<td>50 mM Tris-HCl (pH 8.5)</td>
<td>Tris-HCl (242.28 mg) + MilliQ H₂O (30 mL). pH was adjusted to 8.5 with 10 M NaOH and MilliQ H₂O was added to reach 40 mL.</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Gel</th>
<th>Recipe</th>
</tr>
</thead>
<tbody>
<tr>
<td>2% Agarose gel</td>
<td>agarose (2 g) + 1X TAE (100 mL)</td>
</tr>
<tr>
<td>Buffer</td>
<td>Recipe</td>
</tr>
<tr>
<td>-------------------------------</td>
<td>----------------------------------------------------------------------------------------------------------------------------------------</td>
</tr>
<tr>
<td>Phosphate-Buffer Saline (PBS) 10X (pH 7.4)</td>
<td>sodium chloride (80 g) + potassium chloride (4.2 g) + monopotassium phosphate (2 g) + disodium phosphate (11.5 g) + MilliQ H$_2$O (1000 mL). Diluted to 1X as needed with MilliQ H$_2$O and autoclaved if sterilisation was required. pH was at 7.4 (no adjustment required).</td>
</tr>
<tr>
<td>50 mM NEM (pH 8)</td>
<td>NEM 0.9 g/mL (319.9 µL) + MilliQ H$_2$O (50 mL). pH was adjusted to 8.0 with 1 M HCl.</td>
</tr>
<tr>
<td>Lysis buffer</td>
<td>Tween® (0.125 µL) + proteinase K (0.25 µL)+ 50 mM Tris-HCl (pH 8.5) (49.625 µL). Made fresh each time (50 µL x number of samples)</td>
</tr>
<tr>
<td>50X Tris-acetate-EDTA (TAE) buffer (pH 8.3)</td>
<td>Tris base (242 g) + MilliQ water (750 mL) + acetic acid (57.1 mL) + 500 mM EDTA (pH 8) (100 mL). MilliQ H$_2$O was added to 1 L. Diluted to 1X working solution with MilliQ H$_2$O results in 40 mM Tris, 20 mM acetic acid, 1 mM EDTA.</td>
</tr>
</tbody>
</table>
REFERENCES


(27) Furda AM, Marrangoni AM, Lokshin A, Van Houten B. Oxidants and not alkylating agents induce rapid mtDNA loss and mitochondrial dysfunction. DNA Repair (Amst) 2012 Aug 1;11(8):684-692.


(57) Huang H, Manton KG. The role of oxidative damage in mitochondria during aging: a review. Front Biosci 2004 May 1;9:1100-1117.


87


(78) Moyle G. Toxicity of antiretroviral nucleoside and nucleotide analogues: is mitochondrial toxicity the only mechanism? Drug Saf 2000 Dec;23(6):467-481.


