AN INVESTIGATION INTO 3’-AZIDO-3’-DEOXYTHYMIDINE TOXICITY:

INHIBITION OF THYMIDINE PHOSPHORYLATION

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by

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3’-azido-3’-deoxythymidine (AZT) has been a staple of AIDS therapy for over two decades. Long-term use of high dosage AZT, as seen in the late 1980’s and early 1990’s, was associated with tissue toxicities, including hepatotoxicity and cardiomyopathy. Low dosage AZT therapy used in modern regimens is associated with lipodystrophy. Mitochondria in the affected tissues are dysfunctional and have depleted mitochondrial DNA. The toxicity of AZT is hypothesized to be due to AZT-5’-triphosphate (AZT-TP) inhibition of the mitochondrial DNA polymerase γ, leading to mitochondrial DNA depletion. In this work with
isolated rat liver mitochondria and previous work with isolated rat heart mitochondria, AZT was phosphorylated to AZT-5'-monophosphate (AZT-MP), but no AZT-TP was detectable over two hours of incubation. However, in these mitochondria, AZT was shown to be a potent competitive inhibitor of thymidine phosphorylation. From this work, an alternative mechanism for AZT toxicity was proposed, wherein AZT’s inhibition of thymidine phosphorylation leads to a depletion of the TTP pool, which causes mitochondrial DNA depletion.

The 3T3-F442a cell culture model was used to further investigate this mechanism of toxicity. These cells are mouse pre-adipocytes that can be differentiated into adipocytes, making these cells a good model for lipodystrophy. The 3T3-F442a cells were grown and differentiated in the presence of 1 and 10 µM AZT for 12 days. Samples were analyzed to determine mitochondrial DNA content and deoxynuceloside-triphosphate (dNTP) levels. Both 1 and 10 µM AZT were toxic to mitochondria but caused a significant increase in mitochondrial DNA content relative to untreated cells. This is the opposite of the expected effect and may reflect a compensatory mechanism to overcome toxicity. Also, AZT did not cause any trend of variation from the untreated cells in the dNTP pools during the 12 days of treatment. These results do not provide any
further evidence to advance the proposed mechanism of AZT toxicity due to AZT inhibition of thymidine phosphorylation.
To my parents
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ABBREVIATIONS

3TC  lamivudine, 2’,3’-dideoxy-3’-thiacytidine
ADP  adenosine-5’-diphosphate
AIC  Akaike’s information criterion
AIDS  acquired immunodeficiency syndrome
ATP  adenosine-5’-triphosphate
AZT  zidovudine, 3’-azido-3’-deoxythymidine
AZT-MP  3’-azido-3’-deoxythymidine-5’-monophosphate
AZT-DP  3’-azido-3’-deoxythymidine-5’-diphosphate
AZT-TP  3’-azido-3’-deoxythymidine-5’-triphosphate
CNT  concentrative nucleoside transporter
CPM  counts per minute
d4T  stavudine, 2’,3’-didehydro-3’-deoxythymidine
d4T-TP  2’,3’-didehydro-3’-deoxythymidine-5’-triphosphate
dATP  deoxyadenosine-5’-triphosphate
dCTP  deoxycytidine-5’-triphosphate
ddi  didanosine, 2’,3’-dideoxyinosine
dGTP  deoxyguanosine-5′-triphosphate
DNC  deoxynucleotide carrier
dNTP  deoxynucleoside-5′-triphosphate
ENT  equilibrative nucleoside transporter
HAART  highly active antiretroviral therapy
HIV  human immunodeficiency virus
HPLC  high pressure liquid chromatography
IC_{50}  50% inhibition concentration
K_i  inhibition constant
mtDNA  mitochondrial DNA
nDNA  nuclear DNA
nNRTI  non-nucleoside reverse transcriptase inhibitor
NRTI  nucleoside-analog reverse transcriptase inhibitor
NRTI-TP  nucleoside-analog reverse transcriptase inhibitor triphosphate
PCR  polymerase chain reaction
SEM  standard error of the mean
TDP  thymidine-5′-diphosphate
TMP  thymidine-5′-monophosphate
TTP  thymidine-5′triphosphate
CHAPTER 1:

INTRODUCTION

1.1 Background of AIDS and HIV life cycle

Acquired immunodeficiency syndrome (AIDS) is a worldwide epidemic. This condition is known to be caused by infection with the human immunodeficiency virus (HIV), resulting in a profound depletion of CD4+ T-cells (<200 per µl blood). Since CD4+ T-cells are a key component of innate immunity, patients with AIDS have a severely compromised immune system. This results in many conditions and opportunistic infections, such as Kaposi’s sarcoma and Pneumocystis carinii pneumonia, which are rarely seen in those with a healthy immune system. In most patients, these opportunistic conditions are the eventual cause of death.

HIV is a member of the lentivirus genus of the retroviridae family. The virus is transmitted through contact with infected body fluids, such as blood and semen. Once in the blood stream, HIV interacts with several cell types, including CD4+ T-cells and macrophages, by binding the CD4
receptor on the cells with the viral gp120 protein in the viral envelope (Connor & Ho 1992; Haseltine 1992). The chemokine receptors found on the surface of the T-cell, CCR5 and CXCR4, act as cellular co-receptors, facilitating the fusion of the viral envelope and the cellular plasma membrane and release of the viral core into the cytoplasm. The viral core is then partially uncoated, exposing the viral genome to the cytoplasm and activating the viral reverse transcriptase to convert the HIV RNA genome into DNA, which is then transported into the nucleus. Viral integrase inserts the HIV DNA into the cellular genome. At this point, HIV may enter either a latent state or begin an active infection, generating new viral mRNA and protein. In an active infection, all the necessary proteins for creating new virions are made. The new virion is assembled at the plasma membrane, where two copies of the HIV RNA genome are packaged into the nucleocapsid along with reverse transcriptase and integrase. The virion then buds from the plasma membrane, creating the viral envelope, and releasing it into the extracellular fluid.

1.2 Background on drugs used to combat HIV

Although there is no cure for HIV infection, a variety of pharmaceuticals have been developed to slow the progression to AIDS.
All of these drugs exploit various aspects of the unusual life cycle of HIV. AZT (3’-azido-3’-deoxythymidine, zidovudine) was the first drug approved for the treatment of HIV by the Food and Drug Administration in 1987. Although AZT was originally developed as an anti-cancer compound in the 1960’s, it was never approved for that use (Horwitz et al. 1964; Lin & Prusoff 1978). Researchers later found that AZT inhibited the replication of HIV (Mitsuya et al. 1985). Other compounds similar to AZT, such as d4T (stavudine), 3TC (lamivudine), and ddI (didanosine), have been developed and now constitute the class of drugs known as the nucleoside-analog reverse transcriptase inhibitors (NRTIs) (Gotte et al. 2002). These compounds block the conversion of the HIV RNA genome to DNA by inhibiting the viral reverse transcriptase. NRTIs were given in mono- and dual-therapy regimens until 1996 when two new classes of anti-HIV drugs became available.

In 1996, non-nucleoside reverse transcriptase inhibitors (nNRTIs), such as nevirapine and delavirdine, and protease inhibitors, such as ritonavir and indinavir, were approved for use. nNRTIs, like NRTIs, inhibit the HIV reverse transcriptase (Balzarini 2002). Protease inhibitors, however, function to prevent the viral protease from cleaving the large HIV translation product into its several smaller, active proteins (Condra &
Vacca 2002). In turn, this prevents new virions from assembling and thus stops new cells from being infected. At this time, the modern standard for HIV therapy, called highly active antiretroviral therapy (HAART) began. This is combination therapy typically consisting of two reverse transcriptase inhibitors and one protease inhibitor.

While new drugs have been added to these three classes, no new classes had been approved until 2003 with the introduction of enfuvirtide, a fusion inhibitor that binds to the viral surface protein gp41 and prevents the fusion of the virion with the plasma membrane of the target cell after the binding of gp120 to CD4 (De Clercq 2002). Even though this drug is very effective, it is currently too cost-prohibitive for wide usage. Several other groups of compounds, including integrase inhibitors, coreceptor antagonists, nucleocapsid-targeted agents, and transcription inhibitors, have not yet been approved for use but are being investigated (De Clercq 2002).

1.3 NRTI mechanism of action

As the name suggests, NRTIs are analogs to the naturally occurring deoxynucleosides. These analogs can be added to a replicating DNA strand in place of their natural counterparts. For example, AZT is a
thymidine analog, with the only difference between AZT and thymidine being that AZT has an azido group at the 3’ position on the deoxyribose whereas thymidine has a hydroxyl group. This lack of the 3’-hydroxyl group is a common feature among NRTIs. This allows these molecules to act as DNA strand terminators since all DNA polymerases require the presence of a correctly base-paired nucleotide with a free 3’-hydroxyl group onto which the next nucleotide is added.

Therapeutically, NRTIs must be given in a pro-drug form. The unphosphorylated nucleoside analogs must be activated enzymatically after they are taken up by cells. Unphosphorylated nucleosides are transported across the plasma membrane, whereas phosphorylated nucleosides are not. NRTIs are phosphorylated through the cellular deoxynucleoside salvage pathways to the triphosphate form. In the case of AZT, the thymidine salvage pathways are followed.

1.4 Thymidine salvage pathways

Thymidine is first transported across the plasma membrane into the cytosol. This transport is accomplished by either a concentrative nucleoside transporter (CNT) or equilibrative nucleoside transporter
Figure 1. Overview of thymidine phosphorylation.

Once transported into the cytoplasm, thymidine can be phosphorylated to TMP by thymidine kinase 1 (TK1). Thymidylate kinase (TMPK) phosphorylates TMP to TDP, and nucleoside diphosphate kinase (NDPK) phosphorylates TDP to TTP. Thymidine can be transported into the mitochondrial matrix and phosphorylated by thymidine kinase 2 (TK2) to TMP. A de novo pathway to synthesize TMP is also present in some cell types.
(ENT). Once in the cytosol, the pathway branches (Fig. 1). In the cytosolic branch, thymidine is phosphorylated to TMP by the cytosolic thymidine kinase 1. Thymidylate kinase then phosphorylates TMP to TDP. Finally, a nucleoside diphosphate kinase phosphorylates TDP to TTP.

In the other branch, thymidine is transported into the mitochondrial matrix. While it is clear that this transport occurs, it is not yet certain as to which carrier is actually responsible. The ENT may play a role in humans, but it is not found localized to the mitochondria in rodents (Lai et al. 2004; Lee et al. 2006).

In the matrix, thymidine kinase 2 phosphorylates thymidine to TMP (Fig. 1). TMP is then phosphorylated to TDP. Even though this enzymatic activity has been observed, the particular enzyme that catalyzes it has not yet been characterized. As in the cytosol, TDP is phosphorylated to TTP by a matrix nucleoside diphosphate kinase. It is important to note that TMP does not appear to be transported and must either be further phosphorylated to TDP or TTP, both of which are readily transported, or dephosphorylated to thymidine by a 5’-nucleotidase in order to move into or out of the mitochondrial matrix (McKee et al. 2004). For some time, the deoxynucleotide carrier (DNC) was believed to play a major role in moving deoxynucleotides in and out of the matrix, but recent evidence
suggests that the DNC may actually transport thiamine pyrophosphate primarily (Lewis et al. 2006; Lindhurst et al. 2006).

AZT follows much of the same pathway as thymidine and utilizes the same enzymes (Fig. 2). However, there are some key differences. AZT-MP is a poor substrate for the cytosolic thymidylate kinase (Lavie et al. 1997a; Lavie et al. 1997b). This creates a bottle-neck in the phosphorylation pathway, resulting in only small amounts of AZT-DP and AZT-TP being made. Also, AZT-MP does not appear to be phosphorylated at all within the mitochondrial matrix (McKee et al. 2004) and is a poor substrate for the 5'-nucleotidase (Mazzon et al. 2003), leading to accumulation of AZT-MP in the matrix.

Similar pathways exist to salvage each of the other deoxynucleosides, deoxyadenosine, deoxycytidine, deoxyguanosine, and deoxyuridine. There are only four enzymes that are responsible for the initial phosphorylation to the monophosphate (Johansson et al. 1999; Van Rompay et al. 2000). Two are found in the cytosol and two in the matrix. This design allows any of the five primary deoxynucleosides to be salvaged in either compartment. In the cytosol, thymidine kinase 1 will phosphorylate deoxyuridine as well as thymidine, and deoxycytidine kinase
AZT uses the same enzymes as thymidine. Thymidine kinase 1 (TK1) phosphorylates AZT to AZT-MP in the cytosol. AZT-MP is a poor substrate for thymidylate kinase (TMPK), resulting in little production of AZT-DP. Nucleoside diphosphate kinase (NDPK) will phosphorylate AZT-DP to AZT-TP. AZT can be transported into the matrix and phosphorylated by thymidine kinase 2 (TK2). AZT-MP does not appear to be phosphorylated at all within the matrix.
phosphorylates deoxycytidine and the purines, deoxyadenosine and deoxyguanosine. Within the matrix, thymidine kinase 2 can phosphorylate thymidine, deoxyuridine, and deoxycytidine, and deoxyguanosine kinase can phosphorylate deoxyadenosine and deoxyguanosine. All of these enzymes are constitutively expressed throughout the cell cycle with the exception of thymidine kinase 1, which is expressed only during S phase (Coppock & Pardee 1987). In cells that are not in S phase, thymidine can only be salvaged through the mitochondrial thymidine kinase 2 route.

1.5 Toxicity of AZT

Like all drugs, AZT and the other NRTIs are associated with various adverse reactions. In the late 1980’s and early 1990’s, AZT was given in monotherapy at a relative high dosage of 1200 mg per day. Under this regimen, AZT was associated with skeletal myopathy, cardiomyopathy, hepatotoxicity, various cytopenias, and lactic acidosis (Arnaudo et al. 1991; Benbrik et al. 1997; Chariot et al. 1999; Corcuera et al. 1996; Cupler et al. 1995; Dalakas et al. 1990; Mhiri et al. 1991; Olano et al. 1995; Sinnwell et al. 1995; Tanuma et al. 2003). These adverse effects were serious enough to result in the discontinuation of therapy in 25-30% of patients. In the modern HAART regimen, AZT is given at a lower
dosage of AZT at 600 mg per day and in combination with other drugs. This has resulted in cardiac, muscular, and hepatic adverse effects seen under the monotherapy becoming rare, and now the primary toxic effects are AZT-associated lipodystrophy and anemia (Barile et al. 1998; Deveaud et al. 2005; Moh et al. 2005).

While these affected tissues are quite diverse, they do have a key common feature. They are all high oxygen demand tissues. Additionally, AZT toxicity is often associated with lactic acidosis (Chariot et al. 1999; Hobbs et al. 1995; Lewis et al. 2001; Olano et al. 1995; Pan-Zhou et al. 2000), a sign that certain tissues are unable to generate energy through oxidative phosphorylation and have resorted to anaerobic respiration. These features point to a problem associated with the mitochondria. In fact, mitochondria in AZT-toxic tissues have an abnormal morphology with a reduced number of cristae that suggests a problem with the electron transport chain found imbedded in the inner mitochondrial membrane that makes up the cristae. This problem is a result of AZT-induced mtDNA depletion that is often observed in affected tissues displaying toxicity (Lewis et al. 1992; Lim & Copeland 2001; Martin et al. 1994). Without mtDNA, key components of the electron transport chain cannot be synthesized, causing oxidative phosphorylation to slow or stop.
The current prevailing hypothesis for the toxicity of AZT and the other NRTIs suggests that the NRTI-triphosphate (NRTI-TP) inhibits the mitochondrial DNA polymerase \( \gamma \) (Lewis & Dalakas 1995; Martin et al. 1994). This may occur in a similar fashion to how NRTI-TP inhibits HIV reverse transcriptase. Polymerase \( \gamma \) may insert the NRTI-TP instead of the natural deoxynucleotide into the growing DNA strand, where the NRTI acts as a chain terminator because of its lack of a 3’-hydroxyl group. This, in turn, results in the observed mtDNA depletion.

While this hypothesis may hold true for the other NRTIs, it does not seem likely to be the mechanism behind AZT toxicity. The 50% inhibition concentration (IC\( _{50} \)) for AZT-TP inhibition of mtDNA synthesis is \( >100 \mu M \) (Martin et al. 1994). When compared to the IC\( _{50} \) of other NRTIs, such as d4T-TP at 10 \( \mu M \) and ddC-TP at 0.002 \( \mu M \), AZT-TP appears to be a poor inhibitor. This is compounded by the fact that AZT-MP is a poor substrate for thymidylate kinase, resulting in an insufficient amount of AZT-TP made within any given cell to be significantly inhibitory towards polymerase \( \gamma \) (Lavie et al. 1997a).
1.6 Alternative mechanism for AZT toxicity

Previous work from this laboratory has been done in isolated rat heart mitochondria and in the isolated perfused adult rat heart (McKee et al. 2004; Susan-Resiga et al. 2007). Thymidine is readily phosphorylated to TTP in both of these models, with the major phosphorylated form being TMP in the isolated mitochondria and TTP in the perfused heart. Both models also readily phosphorylate AZT to AZT-MP. However, no AZT-DP or AZT-TP was detectable after two hours of mitochondrial incubation or three hours of heart perfusion. Thymidine kinase 2 is the only enzyme available to phosphorylate thymidine in these model systems since thymidine kinase 1 is only found in the cytosol of cells in S phase (Coppock & Pardee 1985). The cytosol is removed in the preparation of isolated mitochondria, and the adult rat heart consists mainly of non-replicating cells. As thymidine kinase 2 must be phosphorylating both thymidine and AZT, the effect of AZT on thymidine phosphorylation was observed. AZT inhibits thymidine phosphorylation in isolated rat heart mitochondria and in the perfused adult rat heart, with 50% inhibition concentrations of $7.0 \pm 1.0 \mu M$ and $24 \pm 4 \mu M$ respectively (McKee et al. 2004; Susan-Resiga et al. 2007). This is inhibition due to the pro-drug AZT, not AZT-TP. Additionally, these 50% inhibition concentrations are
much closer to the actual serum concentration observed with the AZT monotherapy of the late 1980’s and early 1990’s. The steady-state 1.5 hour post-dose peak serum concentration observed with long-term oral administration of 250 mg of AZT every four hours is 2.32 µM, with a range of 0.19-5.46 µM (Barnhart 1990). While this dosage is slightly higher than the recommended 200 mg every four hours used in AZT monotherapy regimens, both dosages are in the range of dose-independent kinetics. Given this, it is possible that AZT inhibition of thymidine phosphorylation may play a significant role in causing the mitochondrial toxicities observed with long-term AZT therapy regimens.

Non-replicating tissues, such as heart, can only salvage thymidine through thymidine kinase 2, and this may be the only route available for these tissues to maintain their pool of TTP, necessary for replication of the mtDNA. AZT inhibition of thymidine kinase 2 may deplete intracellular TTP (Fig. 3). This may slow mtDNA replication and could cause the observed mtDNA depletion in tissues affected by AZT toxicity. Imbalances in any of the deoxynucleotide pools can result in mtDNA deletions and depletions (Pontarin et al. 2006; Song et al. 2005; Song et al. 2003).
Figure 3. Proposed mechanism of AZT toxicity.

This mechanism is shown for non-replicating cells, which are not expressing thymidine kinase 1 (TK1). All thymidine salvage must pass through thymidine kinase 2 (TK2). AZT inhibition of this step results in decreased production of TMP, which in turn decreases the pools of TDP and TTP. The decrease in TTP may upset the balance between the dNTPs and cause mtDNA depletion.
Thymidine

Cytosol

De Novo

TK<sub>1</sub>

TK<sub>2</sub>

MTDNA

AZT

Inhibition

Thymidine

TMP

TDP

TTP
1.7 Objectives

This work seeks to further understand the mechanism of AZT toxicity. This knowledge may aid in the engineering of new NRTIs with fewer toxic effects. Additionally, other compounds may be created that interfere with the mechanism causing AZT toxicity, reducing their prevalence in therapy. Work is already underway with one such compound, uridine, which has been shown to alleviate adverse effects related to treatment with AZT and other NRTIs (Koch et al. 2003; Venhoff et al. 2005; Walker et al. 2006; Walker & Venhoff 2005; Walker et al. 2003).

1.7.1 Isolated mitochondria

Since all previous work had been done in rat heart, it is important to expand the observation to another tissue, rat liver. This will demonstrate any difference that may occur in mitochondria from different tissue types. This work has characterized the kinetics of thymidine and AZT phosphorylation in isolated rat liver mitochondria and compared these results to the previous results obtained in isolated rat heart mitochondria (McKee et al. 2004). AZT inhibition of thymidine phosphorylation has
previously been observed in isolated rat heart mitochondria (McKee et al. 2004). This work has shown that inhibition also occurred in isolated rat liver mitochondria. AZT inhibition of thymidine phosphorylation was further characterized in both rat heart and liver mitochondria in order to determine the type of inhibition occurring. Data from the isolated mitochondria presented in this work has been published (Lynx et al. 2006; Lynx & McKee 2006).

1.7.2 Cultured cells

Isolated mitochondria are not an effective model system for long-term trials, as they are reliably viable for incubations of only a few hours. This is not sufficient time frame to observe changes associated with AZT toxicity, such as mtDNA depletion. This may also not be enough time to demonstrate any changes in the dNTP pools that may be due to AZT inhibition of thymidine phosphorylation. However, cultured cells can be treated with AZT for several days or weeks at a time, providing an excellent model for long-term trials. This work includes studies in two different cell lines. The H9c2 cells are cardiac myoblasts derived from Rattus norvegicus and were chosen to model the effects of AZT on cardiac and skeletal muscle. The 3T3-F442a cells are pre-adipocytes
derived from *Mus musculus* and can be differentiated into adipocytes, modeling the effects that may cause AZT-induced lipodystrophy.
2.1 Isolation and incubation of rat heart and liver mitochondria

Mitochondria were isolated from male Harlan Sprague Dawley rat heart using the method described in McKee et al (2004). Rat liver mitochondria were isolated using a modification of this same method. In this modified procedure, the liver is not perfused before homogenization, and no nagarse is used. The Lowry protein assay, with BSA as a standard, was used to determine mitochondrial protein (Lowry et al. 1951). The intactness of the mitochondria from each isolation was determined by measuring the respiratory control ratio, as described below. The isolated mitochondria were then incubated at 30°C in a medium containing mitochondria at 4 mg protein/ml in 25 mM MOPS buffer (pH 7.2), 90 mM potassium chloride, 4 mM magnesium sulfate, 5 mM potassium phosphate,
0.4 mM EGTA, 44 mM mannitol, 14 mM sucrose, BSA (1 mg/ml), 2 mM ATP, 20 mM glutamate, and 0.1 mM of the other 19 amino acids. [Methyl-\textsuperscript{3}H]-thymidine and [methyl-\textsuperscript{3}H]-AZT was added to this basic medium at varying concentrations and specific radioactivities, which are detailed in the Results section and in relevant figure legends.

2.2 Measurement of the respiratory control ratio

The respiratory control ratio demonstrates the intactness of the inner mitochondrial membrane by measuring oxygen consumption under two conditions. State 3 respiration is when ADP is present, and the mitochondria are actively using oxygen to synthesize ATP through oxidative phosphorylation. State 4 respiration is when no ADP is present, and the mitochondria are “idling” and consuming only a small amount of oxygen. The ratio of the consumption rate during state 3 versus state 4 is the respiratory control ratio. Mitochondrial preparations with respiratory control ratios less than 4 and 6 for heart and liver respectively were discarded.

Measurement of oxygen consumption is done using a YSI 5300 biological oxygen monitor with a recirculating water heating system at 30°C. A 3 ml buffer solution containing 140 mM KCl, 5 mM KH\textsubscript{2}PO\textsubscript{4}, 20
mM MOPS, 22.5 mM malate, and 90 mM glutamate at pH 7.2 is added to each well and allowed to equilibrate with room air. The oxygen probe is then inserted and 30 µl of isolated mitochondria are added. The baseline oxygen consumption (state 4) is measured. Subsequently, 1.2 µmol of ADP is added and the oxygen consumption measured (state 3). Upon conversion of all ADP to ATP, the oxygen consumption returns to the state 4 rate. The ratio is calculated from the state 3 to the second state 4 rate.

2.3 Detection of mitochondrial phosphorylation of thymidine and AZT by direct precipitation

A 0.2 ml aliquot of the incubation medium was removed at various time points and was mixed with an equal volume of 10% trichloroacetic acid. The mixture was kept on ice for at least 10 minutes and then centrifuged. A 0.35 ml aliquot of the acid-soluble supernatant was removed and neutralized with 350 mg of AG-11A8 resin and 0.21 ml of water. The neutralized extract was filtered and analyzed by HPLC, as described below.

This method yields the total of the nucleoside and nucleotide components found in the medium and in the acid-soluble portion of the
mitochondrial matrix. The rate of appearance of phosphorylated forms of a labeled precursor can be quantitated over time or in relation to substrate concentration. This method does not differentiate between phosphorylation within the matrix and phosphorylation outside of the matrix.

2.4 HPLC analysis

[Methyl-\(^{3}\)H]-thymidine, [methyl-\(^{3}\)H]-AZT, and their phosphorylated intermediates from the neutralized acid-soluble extract described in the previous section were identified and quantitated using reverse-phase HPLC with an Alltech nucleoside/nucleotide column connected to an in-line UV monitor (254 nm) and a flow-through scintillation counter (McKee et al. 2004). The buffers used for HPLC analysis were as follows: 60 mM ammonium phosphate and 5 mM tetrabutylammonium phosphate in water (A) and 5 mM tetrabutylammonium phosphate in methanol (B). Three methods used on the HPLC are described below, and results from typical runs are shown in Figure 4. All methods use a total flow rate of 2 ml/min. For [methyl-\(^{3}\)H]-thymidine, the HPLC method starts at 95% A and 5% B, shifts to 70% A and 30% B over 15 minutes using a slightly concave gradient, holds at 70% A and 30% B for 10 minutes, and finally
Figure 4. HPLC analyses of thymidine and AZT nucleosides and nucleotides in the acid soluble extracts of incubated isolated liver mitochondria.

Thymidine standard (A top) and AZT standard (B top) were separated by HPLC with an in-line UV absorbance detector. The acid soluble extract of a mitochondrial sample after 0 minutes (A middle) and 120 minutes (A bottom) of incubation with [methyl-$^3$H]-thymidine (1 µM, 8000 dpm pmol$^{-1}$) were detected by HPLC and an in-line scintillation counter. The acid soluble extract of a mitochondrial sample after 0 minutes (B middle) and 120 minutes (B bottom) of incubation with [methyl-$^3$H]-AZT (1 µM, 8000 dpm pmol$^{-1}$) were detected by HPLC and an in-line scintillation counter. Data published in Lynx et al. (2006).
drops immediately to 95% A 5% B and holds for 5 minutes to re-equilibrate the column. For [methyl-³H]-AZT with AZT concentrations of less than 10 µM, the HPLC method is the same as for [methyl-³H]-thymidine except that it was held at 70% A and 30% B for 15 minutes, instead of 10. For [methyl-³H]-AZT with AZT concentrations of 10 µM or higher, the HPLC method starts at 80% A and 20% B, shifts to 60% A and 40% B over 15 minutes using a slightly concave gradient, holds at 60% A and 40% B for 10 minutes, and finally drops immediately to 80% A 20% B and holds for 5 minutes to re-equilibrate the column. This variation improved separation of AZT and AZT-MP at high concentrations of AZT.

2.5 H9c2 cell culture

H9c2 cells were obtained from American Type Culture Collection and were grown in Dulbecco’s modified Eagle media (high glucose with L-glutamine, pyridoxine hydrochloride, and 110 mg/l sodium pyruvate; Gibco), 10 ml/l antibiotic/antimycotic (100x; Invitrogen), 1.5 g/l sodium bicarbonate, and 10% fetal bovine serum (Gibco). The cells were cultured at 37°C in 5% CO₂. H9c2 cells were subcultured at 70% confluency in order to prevent differentiation.
2.6 Determination of thymidine concentration in growth media

Fresh growth media (10 ml) unexposed to cells, reflecting the condition of the media at the time of the experiments detailed in the following sections, was lyophilized in order to concentrate it. It was then diluted back to 1 ml. The remainder of this protocol was developed from that described previously (Grem et al. 2001). Briefly, 50 µl of glacial acetic acid and 2 ml of acetonitrile were added to each sample. The samples were then mixed and centrifuged at 2000 x g for 20 minutes. The supernatant was removed and then dried using a Speedvac. Each sample was then resuspended in 0.5 ml water and analyzed using reverse phase HPLC with an in-line UV detector (254 nm) with the method described above. In order to determine thymidine concentration, the samples were compared to a standard curve constructed using dialyzed fetal bovine serum spiked with 0.2 to 50 nmol of thymidine and treated in the same fashion as the samples.

2.7 Detection of thymidine and AZT phosphorylation in H9c2 cells

Flasks of cells were incubated in normal growth media supplemented with thymidine and/or AZT. H9c2 cells were grown to 70% confluency. The media was removed, and the cells then were washed
with phosphate buffered saline. Thymidine and/or AZT was mixed with fresh media, and this media was then added to the growth flask at the beginning of the incubation. At the end of the incubation, the media was removed and saved for later analysis, and 5% trichloroacetic acid was added to the flask. The flask was put on ice and gently rocked for 20 minutes. The acid soluble fraction was pipetted off and centrifuged for 3 minutes. The supernatant was then removed and neutralized with AG-11A8 resin.

The media removed at the end of incubation from the H9c2 flask was mixed with an equal volume of 10% trichloroacetic acid and put on ice for 10 minutes. This was then centrifuged, and the acid soluble supernatant removed and neutralized with AG-11A8 resin.

2.8 3T3-F442a cell culture

3T3-F442a cells were kindly provided by Martine Caron (Universite Pierre et Marie Curie; Paris, France). The basic growth media contains Dulbecco’s modified Eagle media (high glucose with L-glutamine, pyridoxine hydrochloride, and 110 mg/l sodium pyruvate; Gibco), 10 ml/l antibiotic/antimycotic (100x; Invitrogen), 1.5 g/l sodium bicarbonate, 176.4 µM biotin, and 85 µM pantothenic acid. The cells were grown in
basic growth media plus 10% newborn calf serum (Gibco). At 70-80% confluence, the cells were passed by first removing the media, washing with phosphate buffered saline (Gibco), and adding 0.05% trypsin in 0.53 mM EDTA (Invitrogen) to each plate, and incubating the plate at 37°C for 3 minutes. The trypsin was neutralized by adding an equal volume of growth media. This mixture was centrifuged at 300 x g for 5 minutes in order to pellet the cells. The cells were then resuspended in growth media and plated again with a dilution of 1:10.

2.9 3T3-F442a differentiation to adipocytes

Fetal bovine serum (Gibco) and insulin (Sigma) were used as follows in order to differentiate pre-adipocyte 3T3-F442a cells into adipocytes (Caron et al. 2004). Cells were plated at a 1:10 dilution after passing and grown in standard growth media. After two days the media was removed and replaced with basic growth media plus 5% newborn calf serum and 5% fetal bovine serum. Two days later, the media was removed and replaced with basic growth media plus 10% fetal bovine serum and insulin (1 µg/ml media). This media was removed and replaced with fresh media containing insulin every two days. After six days, the media was removed.
and replaced with basic growth media with no added serum or insulin. The cells were maintained in this media until harvested.

2.10 Determination of mtDNA copy number

The plate of cells was harvested by incubation with trypsin for 3 minutes at 37°C. An equal volume of growth media was added to neutralize the trypsin. This mixture was centrifuged at 300 x g for 5 minutes in order to pellet the cells. The total DNA was isolated using the Qiagen DNEasy kit. The samples were run on an ABI 7500 quantitative real time PCR machine. Each PCR reaction was a total of 20 µl and contained 50 ng of sample DNA, 0.5 µM of each of the two primers, 0.2 µM probe, and 10 µl of TaqMan MasterMix (Applied Biosystems). The conditions of the run were an initial incubation at 95°C for 10 minutes followed by 40 cycles of 95°C for 15 seconds and 60°C for 1 minute. The amplified genes were ND4, found on the mouse mitochondrial chromosome, and polymerase γ, found on mouse nuclear chromosome 7. The copy number of mtDNA and nDNA was determined by comparisons to standard curves generated with purified PCR product from each of the amplified genes. These PCR products were isolated using the Qiagen
QIAquick PCR purification kit. The data were expressed as mtDNA copies per nDNA copy.

The primers and probes used were:

- **ND4 (11133-11212)**
  - 5’-CATCATCACCTCTATTCTGCCTAGC-3’
  - 5’-AAGTCTCCGCGGCGATGATTATA-3’
  - 5’-6FAM-CTCCAACCTACGAAACGGATCCACACAC-BHQ1-3’
- **Polymerase γ (1185-1332)**
  - 5’-TTCTCGATACTATGAGCATGCACAT-3’
  - 5’-GCTGGACCATTGGCTTTCC-3’
  - 5’-6FAM-AAAGCGAGGCGAAGTCCCCG-BHQ1-3’

2.11 dNTP assay

The cells were lysed by treatment with 5% trichloroacetic acid for 60 minutes on ice. The acid-soluble fraction was removed and centrifuged in order to pellet cellular debris. The supernatant was then neutralized with AG-11A8 resin. The dNTPs in the neutralized sample were measured using the protocol developed previously (Sherman & Fyfe 1989; Song et al. 2005; Song et al. 2003). The reaction buffer consisted of 0.004 U/µl Klenow fragment, 0.01 mg/ml bovine serum albumin, 13.2 nM template with annealed primer, 0.02 µCi/µl [³H]-dNTP, 100 mM
HEPES, and 10 mM MgCl₂ at pH 7.5. [³H]-TTP (125 Ci/mml; Amersham) was used when measuring dATP, dCTP, and dGTP. [³H]-dATP (70 Ci/mmol; Amersham) was used when measuring TTP. The primer was annealed to each template by mixing equal volumes of primer and template and placing this in a 70°C water bath. The water bath was allowed to cool to room temperature, allowing the template and primer to anneal. The sequences of the templates and primer were:

- **dATP template**
  - 5’-AAATAAATAAATAAATAATGGCGGTGGAGGCGG-3’
- **dCTP template**
  - 5’-AAAGAAAGAAAGAAAGAAAGGGCGGTGGAGGCGG-3’
- **dGTP template**
  - 5’-AAACAAACAAACAAACAAACGGCGGTGGAGGCGG-3’
- **TTP template**
  - 5’-TTATTATTATTATTATTAGCGGTGGAGGCGG-3’
- **Primer**
  - 5’-CCGCCTCCACCGCC-3’

In the assay (Fig. 5), 5 µl of sample or standard and 20 µl of reaction buffer were added into a tube. This tube was incubated in a water bath at 37°C for 20 minutes. At the end of the incubation, 20 µl of the reaction mixture was spotted onto a 2 cm disk of DE81
Figure 5. Schematic of the dNTP assay.

The example shown uses the template for measuring TTP in the sample. The other dNTPs are measured in the same fashion but with a different template sequence. Also $[^3H]$-TTP is used in place of $[^3H]$-dATP when measuring dATP, dCTP, and dGTP.
Template with annealled primer

Klenow adds TTP and $^3$H-dATP (dÂTP) from media. When more TTP is present more $^3$H-dATP is incorporated into oligonucleotides.

Reaction mix is blotted onto DE81 paper and dried, allowing dsDNA to adhere.

After washing, DE81 paper is counted in a liquid scintillation counter.
chromatography paper and allowed to dry. The DNA oligos adhere to the DE81 paper and everything else is washed away with three 10 minute washes with 5% Na₂HPO₄, one 10 minute wash with water, and one 10 minute wash with 95% ethanol. The DE81 paper was allowed to dry, placed in scintillation vials with 5 ml of Betamax scintillation fluid, and counted in the liquid scintillation counter. The CPMs obtained were compared to a standard curve in order to calculate the number of moles of dNTP present in each sample.
CHAPTER 3:

RESULTS

3.1 Removal of nagarse from mitochondrial isolation method

Originally, the liver mitochondria to be used for this work were isolated using nagarse, similar to the described method in McKee et al (2004) for the isolation of rat heart mitochondria. In heart mitochondria preparations, the nagarse proteolytically softened the fibrous heart tissue, making it easier to homogenize. Rat liver tissue homogenizes readily without nagarse, and replacement of the nagarse with a solution containing 220 mM mannitol, 70 mM sucrose, 5 mM MOPS, and 2 mM EGTA resulted in better coupled mitochondria with higher respiratory control ratios, measured as described in Materials and Methods. The mean respiratory control ratio ± SEM for the mitochondria isolated with nagarse was 3.9 ± 0.4, and for mitochondria isolated without nagarse, it
was 4.9 ± 0.2. A two-tailed Student’s t-test was used to compare these means and yielded p = 0.04, implying that the difference between the two means is statistically significant.

3.2 Time course of thymidine and AZT phosphorylation in isolated mitochondria

Prior work from this lab has shown that thymidine phosphorylation in isolated rat heart mitochondria is nearly linear over time (McKee et al. 2004). This time course in heart mitochondria was extended to three hours with more frequent sampling over the course of the incubation, and a similar time course was constructed for isolated rat liver mitochondria (Fig. 6). These trials were conducted with the addition of 1 µM [methyl-\(^3\)H]-thymidine to the media, as described in Materials and Methods. In this time course, TMP is the dominant phosphorylated form of thymidine in both heart and liver mitochondria, with the exception of the early time points in the liver mitochondria incubation where TTP was slightly higher than TMP. The total of all phosphorylated forms of thymidine increases linearly throughout the time course in liver mitochondria, and it increases linearly in heart mitochondria after a delay of approximately 30 minutes.
Figure 6. Time courses of thymidine phosphorylation in isolated rat heart and liver mitochondria.

Isolated rat heart and liver mitochondria were incubated with 1 µM [methyl-³H]-thymidine (8000 dpm pmol⁻¹), and the acid-soluble extracts were separated by HPLC as described in Materials and Methods. In each tissue, data represent the mean of two trials and is expressed as the percentage of the sum of thymidine, TMP, TDP, and TTP (1.04 ± 0.08 pmol µl⁻¹ for heart and 1.04 ± 0.14 pmol µl⁻¹ for liver). The “All TNP” line represents the sum of TMP, TDP, and TTP. Data published in Lynx & McKee (2006).
To construct the time course for AZT phosphorylation in isolated rat liver mitochondria, the isolated mitochondria were incubated with the addition of 1 µM [methyl-³H]-AZT to the incubation media as described in Materials and Methods. As with thymidine, AZT (Fig. 7) has a linear rate of conversion of AZT to AZT-MP. However, unlike thymidine, AZT-MP did not appear to be further phosphorylated, and no AZTDP or AZTTP was detected over the course of the two hour incubation period.

3.3 Kinetics of thymidine and AZT phosphorylation in isolated rat liver mitochondria

Using a range of thymidine concentrations, varying from 0.4 µM to 200 µM, the kinetics of thymidine phosphorylation were characterized. The sum of the measured levels of TMP, TDP, and TTP were used to calculate the velocity of total thymidine phosphorylation (Fig. 8A). The data obtained was first fitted to the classical Michaelis-Menton equation \( v = (V_{\text{max}} \times [S]) / (K_m + [S]) \) that describes single subunit enzyme reactions. A reasonable, but not perfect fit, was obtained, yielding a \( V_{\text{max}} \) of 284 ± 23 pmol mg⁻¹ hr⁻¹ and an apparent \( K_m \) of 12.0 ± 3.5 µM. Thymidine kinase
Figure 7. Time course of AZT phosphorylation in isolated liver mitochondria.

Mitochondria were incubated as described in Materials and Methods with 1 µM [methyl-\(^3\)H]-AZT (8000 dpm pmol\(^{-1}\)). Acid soluble extracts were separated by HPLC and detected by an in-line scintillation counter. Results shown are the mean ± SEM of three to four independent observations. Data published in Lynx et al. (2006).
Figure 8. Kinetics of thymidine phosphorylation in isolated rat liver mitochondria.

Panel A shows the velocity of thymidine phosphorylation versus thymidine concentration (0.4-200 µM). For thymidine concentrations between 0.4 µM and 20 µM, the specific radioactivity was 8000 dpm pmol⁻¹, but thymidine concentrations of 40, 80, and 200 µM had specific radioactivities of 5000, 2500, and 1000 dpm pmol⁻¹ respectively, in order to conserve [methyl-³H]-thymidine. Data from four trials were pooled and shown as the mean ± SEM. The $V_{\text{max}}$ and $K_m$ were calculated by best fit using the Michaelis-Menton equation (Sigma Plot 9.01). Repploting of this same data as the velocity of thymidine phosphorylation versus the velocity divided by the thymidine concentration generates an Eadie-Hofstee plot (B). An approximation of the two parts of the biphasic nature of this plot is shown with the two straight lines. Hill plot (B inset) of the same data yields a Hill coefficient (n) of 0.67. Data published in Lynx et al. (2006).
A

Thymidine Phosphorylation (pmol mg\(^{-1}\) hr\(^{-1}\))

\[ V_{\text{max}} = 284 \pm 23 \text{ pmol mg}\(^{-1}\) hr\(^{-1} \]  
\[ K_{\text{m}} = 12.0 \pm 3.5 \mu\text{M} \]

[Thymidine] (\mu\text{M})

B

Thymidine Phosphorylation (pmol mg\(^{-1}\) hr\(^{-1}\))

\[ n = 0.67 \]

\[ \log (Y / (V_{\text{max}} - Y)) \]  
\[ \log ([\text{Thymidine}]) \]

Thymidine Phosphorylation / [Thymidine] (pmol mg\(^{-1}\) hr\(^{-1}\) \mu\text{M}\(^{-1}\))
2 is thought to be a dimer that displays negative cooperativity (Wang & Eriksson 2000; Wang et al. 1999). An Eadie-Hofstee plot (velocity versus velocity divided by substrate concentration) is an excellent way to look for negative cooperativity. In an enzyme displaying no cooperativity, the Eadie-Hofstee plot will be a straight line with a negative slope. However, the Eadie-Hofstee plot for this data is distinctly biphasic (Fig. 8B), and the data are approximated with two straight lines. The Hill coefficient is another tool to measure cooperativity in an enzyme. An enzyme with no cooperativity has a Hill coefficient equal to 1. Positive cooperativity has a Hill coefficient greater than 1 and less than or equal to the number of subunits. A Hill coefficient less than 1, as seen with this data (Fig. 8B inset), implies negative cooperativity. Both the biphasic Eadie-Hofstee plot and the Hill coefficient of 0.67 are highly suggestive of negative cooperativity, which has been previously observed for thymidine with purified thymidine kinase 2 (Wang et al. 1999).

The kinetics of AZT phosphorylation were determined in the same manner as for thymidine phosphorylation, utilizing AZT concentrations varying from 0.4 µM to 120 µM. The Michaelis-Menton equation yields a $V_{max}$ of $68.8 \pm 2.9$ pmol mg$^{-1}$hr$^{-1}$ and an apparent $K_m$ of $6.3 \pm 1.1$ µM (Fig. 9A). As noted for thymidine, the Eadie-Hofstee plot (Fig. 9B) is biphasic,
Figure 9. Kinetics of AZT phosphorylation in isolated liver mitochondria.

Panel A shows the velocity of AZT phosphorylation versus the AZT concentration (0.4-120 µM). For AZT concentration between 0.4 µM and 8 µM, the specific radioactivity was 8000 dpm pmol^{-1}, but AZT concentrations of 20, 40, 80, and 200 µM had specific radioactivities of 6500, 3500, 2000, and 1200 dpm pmol^{-1} respectively, in order to conserve [methyl-^{3}H]-AZT. Data from three trials were pooled and expressed as the mean ± SEM. The best fit line for the Michaelis-Menton equation (Sigma Plot 9.01) was used to calculate the $V_{\text{max}}$ and $K_{m}$. Eadie-Hofstee plot (B) of the same data with the velocity of AZT phosphorylation plotted against the velocity divided by the AZT concentration. Since the data is biphasic, it can be approximated with 2 straight lines. Hill plot (B inset) is of the same data and yields a Hill coefficient (n) of 0.76. Data published in Lynx et al. (2006).
and the Hill coefficient (Fig. 9B inset) is 0.76. Again, these both suggest negative cooperativity.

3.4 Inhibition of thymidine phosphorylation by AZT in isolated rat liver mitochondria

Previous work with isolated rat heart mitochondria has shown that AZT inhibits the phosphorylation of thymidine (McKee et al. 2004). To see if this holds true for isolated rat liver mitochondria, [methyl-\(^{3}\)H]-thymidine was incubated in the presence of concentrations of AZT ranging from 0 to 200 \(\mu\)M. A constant thymidine concentration of 1 \(\mu\)M, chosen as an approximation of the physiological concentration of thymidine, was used for all samples. The results demonstrate clearly that increasing the AZT concentration causes a decrease in the rate of thymidine phosphorylation, with an IC\(_{50}\) of 14.4 ± 2.6 \(\mu\)M AZT (Fig. 10).

3.5 AZT is a competitive inhibitor of thymidine phosphorylation

Knowing the type of inhibition caused by AZT allows better predictions to be made in how the phosphorylated thymidine pools may be affected and in ways that the inhibition may be overcome. To determine the type of inhibition, isolated rat heart and liver mitochondria
Figure 10. Effect of AZT on thymidine phosphorylation in isolated liver mitochondria.

Mitochondria were incubated with 1 µM [methyl-³H]-thymidine (8000 dpm pmol⁻¹) and unlabeled AZT in concentrations varying from 0 to 200 µM. The acid soluble extracts were separated by HPLC and detected by an in-line scintillation counter. Data from four trials were pooled and expressed as the mean ± SEM. The percentage of phosphorylation observed as compared to the 0 µM AZT control sample from each experiment is plotted against the AZT concentration. AZT inhibits the phosphorylation of thymidine, with an IC₅₀ of 14.4 ± 2.6 µM AZT, as determined by the best fit line of the data (Sigma Plot 9.01). Data published in Lynx et al. (2006).
IC$_{50}$ = 14.4 ± 2.6 μM AZT
were incubated separately with the addition of [methyl-3H]-thymidine (1 to 64 µM) and unlabeled AZT (0 to 20 µM) to the media. All samples were incubated for 2 hours and the amount of thymidine phosphorylation observed was converted to a per hour rate. The data were graphed on Lineweaver-Burk plots (1/velocity versus 1/substrate concentration, Fig. 11). In a Lineweaver-Burk plot, the y-intercept represents the inverse of the $V_{\text{max}}$, and the x-intercept represents the negative inverse of $K_m$. As the AZT concentration increased, the y-intercepts stayed constant, and the x-intercepts increased in both heart and liver mitochondria (Fig. 11). This pattern of increasing $K_m$ with no change in $V_{\text{max}}$ as the concentration of inhibitor is increased suggests that the data best fits the model of competitive inhibition.

This conclusion was further corroborated with additional analysis using Enzyme Kinetics Pro v2.36. The data for AZT inhibition of thymidine phosphorylation were fit to the mathematical models of four types of inhibition, competitive, noncompetitive, uncompetitive, and mixed (Table 1). This analysis revealed that the model for competitive inhibition provides the best fit of the data for both heart and liver mitochondria as demonstrated by having the lowest AIC value (Yamaoka et al. 1978). Using the competitive inhibition model, values of $K_i$ for AZT
Figure 11. Inhibition of thymidine phosphorylation by AZT in isolated rat heart and liver mitochondria.

Isolated rat heart and liver mitochondria were incubated separately for 120 minutes with [methyl-³H]-thymidine and unlabeled AZT. AZT concentrations were 0, 2, 8, and 20 µM, and thymidine concentrations were 1, 2, 4, 8, 16, 32, and 64 µM. The specific radioactivities were 8000 dpm pmol⁻¹ for samples containing 1, 2, 4, and 8 µM [methyl-³H]-thymidine, and 5600, 2800, and 1400 dpm pmol⁻¹ for samples containing respectively 16, 32, and 64 µM [methyl-³H]-thymidine. The rate of thymidine phosphorylation in the acid soluble extracts of the incubated mitochondria was determined as described in Materials and Methods. Data from three independent trials in each tissue are represented as the mean ± SEM. The lines represent the best fit for the data from each tissue to the global equation for linear competitive inhibition. Inverse of the y-intercept is the apparent $V_{max}$, and negative inverse of the x-intercept is the apparent $K_m$. Data published in Lynx & McKee (2006).
TABLE 1

BEST FIT ANALYSIS FOR AZT INHIBITION OF THYMIDINE PHOSPHORYLATION IN ISOLATED MITOCHONDRIA TO THE MATHEMATICAL MODELS OF INHIBITION

<table>
<thead>
<tr>
<th>Inhibition Model</th>
<th>Heart Mitochondria AIC</th>
<th>Liver Mitochondria AIC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Competitive</td>
<td>196.4</td>
<td>249.4</td>
</tr>
<tr>
<td>Noncompetitive</td>
<td>291.3</td>
<td>∞ (no convergence)</td>
</tr>
<tr>
<td>Uncompetitive</td>
<td>302.3</td>
<td>352.1</td>
</tr>
<tr>
<td>Mixed</td>
<td>198.0</td>
<td>∞ (no convergence)</td>
</tr>
</tbody>
</table>

Inhibition were calculated to be 10.6 ± 4.5 µM AZT for heart mitochondria and 14.0 ± 2.5 µM AZT for liver mitochondria.

The data were also analyzed with respect to the phosphorylation of TMP to TDP and of TDP to TTP. AZT does not appear to have any effect on either of these reactions, suggesting that in isolated heart and liver mitochondria, AZT only inhibits the phosphorylation of thymidine to TMP.
3.6 Thymidine concentration in growth media

Some thymidine is present in fetal bovine serum. In order to obtain more accurate measures of specific radioactivities in later experiments, this concentration needed to be determined. In H9c2 media, the thymidine concentration is 0.40 ± 0.01 \( \mu \text{M} \) as determined by the protocol described in Materials and Methods. This data is expressed as the mean ± SEM of four samples.

3.7 Time courses of thymidine and AZT phosphorylation in H9c2 cells

Flasks of H9c2 cells at 80% confluence were incubated for up to 90 minutes in the presence of growth media supplemented with 1.4 \( \mu \text{M} \) \[^{3}\text{H}]\)-thymidine. Thymidine was readily phosphorylated to TTP, reaching half the maximum rate of phosphorylation at 5 minutes and the plateau after 20 minutes (Fig. 12A). The amount of TMP was negligible at all time points, and TDP was not detectable at any time point. No phosphorylated thymidine was detected in the media, and the amount of thymidine present in the media did not drop significantly over the time course (Fig. 12B). The amount of thymine, a thymidine degradation product, present in the media did not change through the time course.
Figure 12. Time course of thymidine phosphorylation in H9c2 cells.

Flasks of cells were incubated for up to 90 minutes in the presence of growth media with a total concentration of 1.4 µM [methyl-³H]-thymidine. Samples were prepared as described in Materials and Methods and analyzed by reverse phase HPLC with an in-line scintillation counter. Data represent the mean ± SEM of three to four independent trials. Cellular extract (A) is shown as pmol per 10⁶ cells versus minutes of incubation, and media (B) is shown as pmol per µl versus minutes of incubation.
Flasks of cells were incubated for up to 48 hours with the addition of 1 µM [\(^3\)H]-AZT to the growth media. H9c2 cells phosphorylate AZT to AZTMP (Fig 13A). AZT-DP and AZT-TP are also produced, albeit at a slower rate. Like for thymidine, the amount of AZT in the media did not change significantly over time (Fig. 13B). Very small amounts of AZT-MP were detected in the media, possibly due to cell lysis.

3.8 Effect of AZT on thymidine phosphorylation in H9c2 cells

Flasks of cells were incubated for 10 minutes in growth media with \([\(^3\)H]-thymidine (1.4 µM) and with unlabeled AZT (0-200 µM). AZT inhibited thymidine phosphorylation in H9c2 cells with a IC\(_{50}\) 21.9 ± 4.1 µM AZT (Fig. 14). TTP was the only phosphorylated form of thymidine observed.

Although AZT inhibited thymidine phosphorylation in H9c2 cells, no effects were observed on cell growth (data not shown). In preliminary trials to measure mtDNA content, no change was observed when H9c2 cells were treated with AZT (data not shown). Since this cell line did not respond in any way to treatment with AZT, no further studies were conducted using H9c2 cells.
Figure 13. Time course of AZT phosphorylation in H9c2 cells.

Flasks of cells were incubated for up to 48 hours with the addition of 1 µM [methyl-³H]-AZT to the growth media. Samples were prepared as described in Materials and Methods. Data represent the mean ± SEM of three to four trials. Cellular extract (A) shown as pmol per 10⁶ cells versus hours of incubation, and media (B) is shown as pmol per µl versus minutes of incubation.
Figure 14. Effect of AZT on thymidine phosphorylation in H9c2 cells.

Flasks of cells were incubated in growth media with a total 1.4 µM [methyl-³H]-thymidine concentration and with unlabeled AZT (0-200 µM). Cellular extracts were prepared as described in Materials and Methods. Data represent the mean ± SEM of three to four trials, shown as the percent of the 0 µM AZT control versus AZT concentration. AZT inhibits thymidine phosphorylation with an IC₅₀ of 21.9 µM AZT, as calculated by the best fit line (SigmaPlot 9.01).
IC₅₀ = 21.9 ± 4.1 µM AZT
3.9 Effect of AZT and d4T on mtDNA content in 3T3-F442a cells

3T3-F442a cells provide a model for NRTI-induced lipodystrophy, a condition known to be caused by AZT and d4T. Work by other groups has shown that AZT is toxic to these cells (Caron et al. 2004; Walker et al. 2006), providing a better model system in which to observe the effects of AZT on mtDNA content and on dNTP pool balance (see below). 3T3-F442a cells were grown and differentiated over the course of 12 days in the presence of 1 or 10 µM AZT or 1 or 10 µM d4T. The fresh NRTI was added to the media each time the media was changed. Samples were taken every two days starting on Day 4, and total DNA was isolated from the cells and the mtDNA to nDNA ratio was determined as described in Materials and Methods. In the untreated control samples, the average number of mtDNA copies per nDNA copy decreased over time from 75.7 ± 6.6 on Day 4 to 35.2 ± 1.3 on Day 12 (Fig. 15 top). This same general pattern of decreasing mtDNA content of time was also observed in all the treated samples. Therefore, the treated samples were expressed as a percentage of the average control value for that day in order to more easily visualize trends. Cells treated with both 1 and 10 µM AZT had a
Figure 15. Effect of AZT and d4T on mtDNA content in 3T3-F442a cells.

The number of mtDNA copies per nDNA copy in the control (top), 1 and 10 µM AZT treated (middle), and 1 and 10 µM d4T treated (bottom) cells was determined by real time PCR as described in Materials and Methods. Data represent the mean ± SEM of three independent trials. The control (top) is shown as absolute mtDNA copies per nDNA copy on each day. The AZT and d4T treated samples (middle and bottom) are calculated as the percentage of the control value for that day. The dotted lines on the middle and bottom panels represent the 100% control level. Statistical significance of the difference from the control (*) was calculated for each entire treated group versus the entire control group using a two-tailed Student’s t-test (p < 10⁻⁷). Treatment with 1 and 10 µM AZT results in a significant increase in mtDNA content relative to the control, while 10 µM d4T results in a significant decrease.
statistically significant increase in mtDNA copy number relative to the control, with $p < 1.5 \times 10^{-8}$ for both treated groups compared as a whole to the control group (Fig. 15 middle). This increase occurs by Day 4 and is maintained through Day 12. In contrast, cells treated with 1 $\mu$M d4T showed no change relative to the control, but treatment with 10 $\mu$M d4T resulted in a statistically significant decrease relative to the control, with $p < 9 \times 10^{-7}$ (Fig. 15 bottom). The decrease first occurs at Day 6 and is maintained through Day 12.

3.10 Effect of AZT and d4T on dNTP Pools in 3T3-F442a cells

Over the course of 12 days, 3T3-F442a cells were differentiated and grown in the presence of 1 or 10 $\mu$M AZT or 1 or 10 $\mu$M d4T. Samples were taken every two days starting on Day 2, and the dNTPs were isolated and measured as described in Materials and Methods. The total number of pmol of all dNTPs present tends to generally increase over the time course, peaking at Day 8 and then falling off again (Fig. 16). Lower total dNTP amounts than the control were observed in the 1 and 10 $\mu$M AZT and 10 $\mu$M d4T samples. These treatments had fewer cells per sample, which may account for some of the difference in total dNTP observed. Due to the variation in total dNTP, the individual dNTP
Figure 16. Time course of total dNTP content in 3T3-F442a cells.

Cell were grown and differentiated in the presence of 1 or 10 µM AZT or 1 or 10 µM d4T or untreated (control). Samples were taken every two days, and dNTPs present in each sample was determined using the assay described in Materials and Methods. Data, mean ± SEM of three independent trials, represent the total amount of dNTP (pmol) in each sample, determined by the sum of the amounts of dATP, dCTP, dGTP, and TTP in each sample. The number of cells in each treatment group was not the same as AZT and d4T inhibited growth, mostly likely causing the variation between the groups.
pools are shown as a percentage of the total dNTP pool for that treatment on that day. The same general trends appear in the control (Fig. 17) and all the treatment groups (Table 2). Through the time course, the percentage of TTP tends to decrease while the percentage of dATP tends to increase. Overall, dCTP and dGTP vary less than dATP and TTP. The effect of AZT and d4T on the dNTP pools is minimal. Some points do show a statistically significant difference from the control (p < 0.05). However, these differences do not show any general trends, with one exception. Treatment with 10 μM d4T cause an increase in the percentage of TTP on Days 4-8.

At Day 10, the media was replaced with one containing no added serum, according to the protocol from which the culture technique was based. Removing the serum eliminates the cells’ ability to salvage deoxynucleosides since the deoxynucleosides normally supplied by the serum are no longer present. This forces the cells to rely entirely on the reserves they have left and the de novo pathway to maintain the dNTP pools. As such, the control and all treatments demonstrate a decrease in the total dNTP pool size from Day 10 to Day 12. This also results in some disruption of the compositions of the total dNTP pool. In particular,
Figure 17. Time course of individual dNTP levels in untreated 3T3-F442a cells.

Cells were grown and differentiated, and samples were taken every two days and analyzed as described in Materials and Methods. Data represent the mean ± SEM of three independent trials expressed as the percentage of the total amount of dNTP present in that sample.
Table 2

Time course of dNTP pools in 3T3-F442A cells treated with AZT and D4T

<table>
<thead>
<tr>
<th></th>
<th>Day 2</th>
<th>Day 4</th>
<th>Day 6</th>
<th>Day 8</th>
<th>Day 10</th>
<th>Day 12</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Percent Total dNTP</td>
<td>Percent Total dNTP</td>
<td>Percent Total dNTP</td>
<td>Percent Total dNTP</td>
<td>Percent Total dNTP</td>
<td>Percent Total dNTP</td>
</tr>
<tr>
<td>Control</td>
<td>14.04 ± 2.11%</td>
<td>15.77 ± 1.44%</td>
<td>15.10 ± 1.47%</td>
<td>32.28 ± 1.94%</td>
<td>32.57 ± 0.89%</td>
<td>35.14 ± 1.80%</td>
</tr>
<tr>
<td>1 µM AZT</td>
<td>16.62 ± 2.81%</td>
<td>17.02 ± 3.05%</td>
<td>19.83 ± 2.65%</td>
<td>24.95 ± 1.93%</td>
<td>27.55 ± 4.55%</td>
<td><strong>23.73 ± 2.78%</strong></td>
</tr>
<tr>
<td>10 µM AZT</td>
<td>22.72 ± 4.77%</td>
<td>15.14 ± 2.55%</td>
<td>21.39 ± 1.94%</td>
<td>26.56 ± 4.75%</td>
<td>30.75 ± 2.66%</td>
<td><strong>17.98 ± 1.90%</strong></td>
</tr>
<tr>
<td>1 µM D4T</td>
<td>12.40 ± 2.46%</td>
<td>14.86 ± 1.45%</td>
<td>15.98 ± 2.82%</td>
<td>30.56 ± 0.49%</td>
<td>32.61 ± 1.97%</td>
<td>29.82 ± 3.24%</td>
</tr>
<tr>
<td>10 µM D4T</td>
<td>11.33 ± 1.23%</td>
<td>15.58 ± 1.58%</td>
<td>17.69 ± 2.55%</td>
<td><strong>23.60 ± 1.30%</strong></td>
<td>27.75 ± 2.68%</td>
<td>31.93 ± 9.00%</td>
</tr>
<tr>
<td>Control</td>
<td>20.02 ± 3.93%</td>
<td>22.67 ± 2.64%</td>
<td>24.41 ± 1.05%</td>
<td>12.31 ± 1.63%</td>
<td>16.58 ± 2.42%</td>
<td>15.11 ± 3.04%</td>
</tr>
<tr>
<td>1 µM AZT</td>
<td>15.79 ± 4.88%</td>
<td>19.14 ± 2.86%</td>
<td>22.56 ± 2.89%</td>
<td>15.69 ± 1.76%</td>
<td>12.40 ± 2.77%</td>
<td>5.38 ± 1.81%</td>
</tr>
<tr>
<td>10 µM AZT</td>
<td>10.82 ± 2.38%</td>
<td>14.06 ± 0.83%</td>
<td><strong>17.83 ± 1.48%</strong></td>
<td>10.33 ± 1.19%</td>
<td>10.38 ± 1.28%</td>
<td>4.10 ± 3.46%</td>
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<tr>
<td>1 µM D4T</td>
<td>15.54 ± 5.35%</td>
<td>17.25 ± 2.88%</td>
<td>22.01 ± 2.27%</td>
<td>13.54 ± 2.75%</td>
<td>14.46 ± 2.53%</td>
<td>14.83 ± 2.83%</td>
</tr>
<tr>
<td>10 µM D4T</td>
<td>18.84 ± 3.15%</td>
<td>13.21 ± 4.21%</td>
<td>14.51 ± 3.41%</td>
<td>8.87 ± 3.20%</td>
<td>12.97 ± 5.71%</td>
<td>8.81 ± 10.82%</td>
</tr>
<tr>
<td>Control</td>
<td>12.13 ± 0.71%</td>
<td>13.94 ± 1.86%</td>
<td>9.77 ± 0.35%</td>
<td>15.55 ± 0.55%</td>
<td>15.26 ± 1.10%</td>
<td>15.52 ± 0.68%</td>
</tr>
<tr>
<td>1 µM AZT</td>
<td>13.06 ± 1.54%</td>
<td>10.47 ± 0.99%</td>
<td>11.15 ± 0.64%</td>
<td>12.18 ± 1.17%</td>
<td>15.12 ± 1.78%</td>
<td>16.18 ± 1.46%</td>
</tr>
<tr>
<td>10 µM AZT</td>
<td><strong>18.41 ± 1.60%</strong></td>
<td>17.92 ± 0.22%</td>
<td>15.63 ± 1.48%</td>
<td>15.82 ± 0.53%</td>
<td>14.89 ± 0.91%</td>
<td><strong>21.50 ± 0.77%</strong></td>
</tr>
<tr>
<td>1 µM D4T</td>
<td>13.17 ± 0.20%</td>
<td>10.57 ± 0.92%</td>
<td>8.81 ± 0.59%</td>
<td><strong>12.13 ± 0.51%</strong></td>
<td>14.28 ± 0.37%</td>
<td>13.89 ± 1.76%</td>
</tr>
<tr>
<td>10 µM D4T</td>
<td>10.37 ± 1.44%</td>
<td>9.02 ± 0.68%</td>
<td>7.84 ± 1.66%</td>
<td><strong>10.10 ± 1.23%</strong></td>
<td>13.63 ± 0.31%</td>
<td><strong>21.50 ± 0.75%</strong></td>
</tr>
<tr>
<td>Control</td>
<td>53.82 ± 4.33%</td>
<td>47.62 ± 2.52%</td>
<td>50.67 ± 2.09%</td>
<td>39.87 ± 2.80%</td>
<td>35.56 ± 2.76%</td>
<td>34.23 ± 2.86%</td>
</tr>
<tr>
<td>1 µM AZT</td>
<td>54.54 ± 2.87%</td>
<td>53.37 ± 5.15%</td>
<td>46.66 ± 4.81%</td>
<td>47.19 ± 2.88%</td>
<td>44.92 ± 1.53%</td>
<td><strong>54.70 ± 2.01%</strong></td>
</tr>
<tr>
<td>10 µM AZT</td>
<td>48.04 ± 2.67%</td>
<td>52.87 ± 3.40%</td>
<td>45.16 ± 1.27%</td>
<td>47.18 ± 4.22%</td>
<td>43.99 ± 1.55%</td>
<td><strong>56.40 ± 1.38%</strong></td>
</tr>
<tr>
<td>1 µM D4T</td>
<td>58.88 ± 3.56%</td>
<td>57.32 ± 2.57%</td>
<td>53.20 ± 1.76%</td>
<td>43.75 ± 3.53%</td>
<td>38.65 ± 1.31%</td>
<td>41.46 ± 3.21%</td>
</tr>
<tr>
<td>10 µM D4T</td>
<td>59.46 ± 3.98%</td>
<td><strong>62.19 ± 3.39%</strong></td>
<td><strong>59.96 ± 1.14%</strong></td>
<td><strong>57.42 ± 1.46%</strong></td>
<td>45.66 ± 4.11%</td>
<td>37.76 ± 2.17%</td>
</tr>
</tbody>
</table>

1 Data is the mean ± SEM of three independent trials, expressed as the percentage of the total for that sample on that day. *Significantly different from the untreated control for that day (p < 0.05), as determined by a two-tailed Student’s t-test.
dATP drops in the 10 µM AZT group, dCTP drops in the 1 and 10 µM AZT groups, dGTP rises in the 10 µM AZT and 10 µM d4T groups, and TTP in the 1 and 10 µM AZT groups.
During the pre-HAART era, AZT monotherapy in AIDS patients was closely associated with various tissues toxicities that included myopathy, cardiomyopathy, and hepatotoxicity (Arnaudo et al. 1991; Benbrik et al. 1997; Chariot et al. 1999; Corcuera et al. 1996; Cupler et al. 1995; Dalakas et al. 1990; Mhiri et al. 1991; Olano et al. 1995; Sinnwell et al. 1995; Tanuma et al. 2003). In these tissues, the toxicity of AZT was correlated with abnormal mitochondria and mtDNA depletion (Lewis et al. 1992; Lim & Copeland 2001). The current prevailing hypothesis suggests that NRTI toxicity is caused by NRTI-TP inhibition of the mitochondrial DNA polymerase $\gamma$ (Lewis & Dalakas 1995; Martin et al. 1994). While this may be a viable mechanism for the other NRTIs, it is unlikely that AZT toxicity occurs in this manner. The measured IC$_{50}$ for AZT-TP inhibition of mtDNA synthesis was $>100$ $\mu$M, a much higher concentration than other NRTIs (Martin et al. 1994). Additionally, AZT is readily phosphorylated to AZT-MP, but AZT-MP is a poor substrate for thymidylate kinase, leading to little production of AZT-TP (Lavie et al. 1997a). Taken together, these
factors imply that another mechanism must be behind the toxic side
effect of AZT therapy.

Earlier work from this laboratory investigated the ability of isolated
rat heart mitochondria to phosphorylate thymidine and AZT (McKee et al.
2004). The findings described here extend these studies to isolated rat
liver mitochondria. Comparing the liver mitochondria studies with those
from heart mitochondria demonstrates that mitochondria from both
tissues readily phosphorylate thymidine to TTP. In both heart and liver,
TMP was the predominant phosphorylated form of thymidine. This
suggests that the thymidylate kinase reaction, which converts TMP to
TDP, has become the rate-limiting reaction in the pathway in isolated
mitochondria. The kinetics of the initial reaction of the conversion of
thymidine to TMP were determined in both tissues. In the liver
mitochondria, the $V_{\text{max}}$ of this reaction was about seven fold faster, and
the $K_m$ was about six fold higher. The kinetic data in both heart and liver
mitochondria were treated as an Eadie-Hofstee plot. For a typical enzyme
that displays no cooperativity, an Eadie-Hofstee plot gives a straight line
with a negative slope and the slope is the negative of the $K_m$ value. The
kinetic data from liver mitochondria and from heart mitochondria are
distinctly biphasic. This shape to the Eadie-Hofstee plot implies that the
enzyme catalyzing the reaction displays negative cooperativity. The determined values of the Hill coefficient are also consistent with negative cooperativity. A model for negative cooperativity is when binding of substrate at the first site lowers the affinity of binding at the second site. Very few enzymes have displayed this unusual characteristic. Work with purified thymidine kinase 2 has shown that this enzyme displays negative cooperativity, while thymidine kinase 1 does not (Wang et al. 1999).

Hence, the presence of negative cooperativity in the thymidine phosphorylation reaction in isolated mitochondria serves as an identifying marker that thymidine kinase 2 was the most likely enzyme to be phosphorylating thymidine in isolated rat heart and liver mitochondria.

AZT was readily phosphorylated to AZT-MP in mitochondria from both tissues. No AZT-DP or AZT-TP was detected over the course of the incubation in either heart or liver mitochondria. This may be due to the limited time frame in which isolated mitochondria may be studied or to poor reactivity of AZT-MP with the mitochondrial thymidylate kinase, as with the cytosolic thymidylate kinase (Lavie et al. 1997a). The liver mitochondria displayed a $V_{\text{max}}$ for AZT phosphorylation about three fold faster than heart mitochondria. However, unlike thymidine, the $K_m$ was nearly the same as isolated mitochondria from both tissues. Liver and
heart mitochondria both display negative cooperativity in the phosphorylation of AZT, suggesting that AZT is also being phosphorylated by thymidine kinase 2.

Since the same enzyme in isolated rat liver mitochondria appears to phosphorylate both thymidine and AZT, it seemed possible that AZT would have an inhibitory effect on thymidine phosphorylation, as seen in previous work with isolated rat heart mitochondria (McKee et al. 2004). The IC$_{50}$ for AZT inhibition of thymidine phosphorylation in liver mitochondria was determined to be 14.4 ± 2.6 µM AZT. This value is similar to the IC$_{50}$ of 7.0 ± 1.0 µM AZT observed for heart mitochondria (McKee et al. 2004). This inhibition was further characterized in both heart and liver mitochondria. Both tissues follow the same kinetic pattern treated as Lineweaver-Burl plots. The x-intercepts (-1/K$_m$) increase and the y-intercepts (1/V$_{max}$) stay constant as the concentration of AZT is raised, leading to the conclusion that AZT is a competitive inhibitor of thymidine phosphorylation in isolated rat heart and liver mitochondria. Mathematical analysis of the data confirms that AZT is a competitive inhibitor.

This work provides additional support to the alternative mechanism of toxicity proposed in prior work from this laboratory (McKee et al.}
In this mechanism, AZT competitively inhibits thymidine kinase 2 in non-replicating tissues, such as heart and liver. Since thymidine kinase 1 is only expressed during S phase of the cell cycle (Coppock & Pardee 1987), thymidine kinase 2 is the only route for non-replicating tissue to salvage and phosphorylate thymidine. AZT, by acting to inhibit thymidine kinase 2, may greatly slow the conversion of thymidine to TMP. A reduction in the production of TMP could result in a reduction in TTP, as TMP is a necessary precursor to TTP. As TTP becomes limiting, mitochondrial DNA may slow replication and eventually lead to mtDNA depletion. It has been shown that imbalances in any of the dNTP pools will cause mtDNA mutations, deletions, and depletion (Pontarin et al. 2006; Song et al. 2005; Song et al. 2003). Therefore, an imbalance in the TTP pool created by AZT inhibition of thymidine phosphorylation could account for the mtDNA depletion observed with AZT toxicities.

Additional strong support for this mechanism of toxicity comes from the inherited partial deficiency of thymidine kinase 2. In the few cases of this rare human genetic disorder that have been characterized, the patients have presented with a severe mitochondrial myopathy with mtDNA depletion and have died in early childhood (Saada et al. 2003b; Saada et al. 2001). Saada et al. have demonstrated that TTP is
significantly reduced in fibroblast cells cultured from these patients (Saada et al. 2003a). Liver damage has not been reported in the few individuals who have been characterized to have a partial thymidine kinase 2 deficiency; however, a similar mtDNA depletion disease affecting liver and brain has been characterized in which the matrix enzyme deoxyguanosine kinase is partially deficient (Mandel et al. 2001). This enzyme is involved in the salvage of deoxyguanosine, deoxyadenosine, and deoxyinosine by phosphorylation of these compounds to their monophosphates. The difference in the presenting pathology of these two disorders is unknown, but it may be related to tissue-specific levels of expression of the respective enzymes.

In order to further advance this mechanism for AZT toxicity, it will be necessary to conduct long-term trials. The studies thus far represent only short-term changes and do not reflect how a living cell would react to and possibly compensate for AZT inhibition of thymidine phosphorylation. Once purified from a tissue, isolated mitochondria are only viable for a few hours. This is not sufficient time to observe any changes in the mtDNA content, making isolated mitochondria a poor model for long-term effects of AZT toxicity.
One option for long-term trials is cultured cells. The cells can be grown in the presence of an NRTI and the effects observed over the course of days or weeks. A variety of cell lines have been used in NRTI toxicity studies. This work includes studies in the H9c2 cell line, cardiac myoblasts derived from *Rattus norvegicus*, and the 3T3-F442a cell line, pre-adipocytes derived from *Mus musculus*.

H9c2 cells were able to salvage and phosphorylate thymidine and AZT. There are some differences from isolated mitochondria. One is that TTP, not TMP, was the dominant phosphorylated form of thymidine. This is logical since TTP is the active form of thymidine needed for DNA replication and repair, whereas TMP is a precursor. In the isolated perfused rat heart, TTP was also the major phosphorylated form of thymidine (Susan-Resiga et al. 2007). Like cell culture, the isolated perfused rat heart is a model system utilizing intact cells. The prevalence of TMP in isolated mitochondria may be due to the inability of an *in vitro* system to maintain the very high energy charge obtained by intact cells. Alternatively, an interaction with a component found outside of the mitochondria may be necessary in order to shift the equilibrium from TMP to TTP in the isolated mitochondria system.
Another interesting difference is that unlike isolated mitochondria, H9c2 cells do produce some AZT-TP. Nevertheless, it is a small amount (~8 pmol/10⁶ cells), and the level appears to plateau after 12 hours. However, it is important to note that the H9c2 cells are growing and actively replicating their DNA. Therefore, there will be a subpopulation in S phase that will be expressing the cytosolic thymidine kinase 1. The cytosolic pathway can produce AZT-TP, albeit in small amounts as seen here. These low concentrations of AZT-TP are sufficient to inhibit HIV reverse transcriptase but are too low to block mtDNA synthesis through inhibition of polymerase γ (Martin et al. 1994).

Like in isolated mitochondria, AZT inhibits thymidine phosphorylation in H9c2 cells. This was measured as a reduction in the amount of thymidine phosphorylated and did not discriminate between cytosolic and mitochondrial phosphorylation. Thus, this inhibition represents not only thymidine kinase 2 but also thymidine kinase 1.

Unlike isolated mitochondria, we can conduct long-term experiments with this cultured cell line by growing the cells in the presence of AZT of an extended period of time. Preliminary trials (data not shown) showed that AZT did not appear to affect the rate of growth of H9c2 throughout any of the trials conducted. Further, AZT had no
effect on mtDNA content of H9c2 cells (data not shown). Personal communications with Kendall Wallace and a later publication from his group confirmed our observations (Lund et al. 2007). When treated with up to 50 µM AZT, H9c2 cells showed no significant change in mtDNA content. Even though AZT inhibits thymidine phosphorylation in this cell line, this inhibition does not correlate with mtDNA depletion, or even toxicity, in H9c2 cell populations. A possible explanation for this may be that H9c2 cells have an active de novo pathway for synthesis of deoxynucleotides. This would reduce the importance of salvage enzymes and allow a cell to grow unabated even when the salvage pathways are inhibited completely. While this cell line initially appeared promising because of its cardiac-like properties, H9c2 cells do not show any toxic effects when exposed to AZT and serve as a poor model for determining the mechanism of AZT toxicity.

One of the more common and severe adverse effects of HAART therapy today is lipodystrophy (Caron et al. 2004; Deveaud et al. 2005; Moyle 2000; Tozser 2001), characterized by loss of the peripheral adipose tissue with concurrent accumulation of fat in the central adipose. This results in very thin arms and legs and wasting in the face, often with sunken eyes and cheeks, along with a round, swollen abdomen and buffalo
hump. These effects can be severe and disfiguring to the point that reconstructive surgery has been used as an attempt to reverse the wasted appearance in the face (Domergue et al. 2006; Negredo et al. 2006). A variety of AIDS drugs have been associated with lipodystrophy, including the protease inhibitors and some NRTIs, such as AZT and d4T. The 3T3-F442a cell line provides a good model to study lipodystrophy. These cells are pre-adipocytes that can be induced to differentiate into adipocytes. Additionally, 3T3-F442a cells have been shown to be sensitive to treatment with various NRTIs (Caron et al. 2004; Walker et al. 2006).

Our proposed mechanism for AZT toxicity suggests that AZT inhibits thymidine phosphorylation, resulting in a depletion of the TTP pool. The imbalance of TTP compared to the other dNTPs causes mtDNA depletion. On the other hand, d4T is much more potently toxic to the polymerase γ, with an IC_{50} for d4T-TP inhibition of mtDNA synthesis of 10 μM (Martin et al. 1994), and d4T has not demonstrated any inhibitory effects on thymidine phosphorylation in the isolated perfused heart (Susan-Resiga et al. 2007) or in isolated mitochondria (McKee unpublished data). Unlike in H9c2 cells, AZT and d4T have been shown to be toxic to mitochondria in the 3T3-F442a cells. In these trials with the
3T3-F442a cell line, d4T was used to demonstrate the effects of an NRTI causing toxic effects through the traditional polymerase $\gamma$ inhibition model. This provides a ready comparison to the effects of AZT that may be utilizing a different mechanism of toxicity.

In order to evaluate the effects of AZT and d4T on 3T3-F442a cells, the mtDNA content was measured. Depletion of the mtDNA was seen in the group of cells treated with 10 $\mu$M d4T that confirms results previously obtained (Walker et al. 2006). No effect was observed when treating with 1 $\mu$M d4T. However, both 1 and 10 $\mu$M AZT resulted in an increase in mtDNA content. This is contrary to what was found by Walker et al. (2006), who observed mtDNA depletion at 1 $\mu$M AZT. It is unclear why the AZT results differ. Our starting stock of 3T3-F442a was obtained from Walker et al., and our protocol is based on theirs.

AZT has been shown to cause an increase in mtDNA content in other cell lines as well, such as HepG2 (Lund et al. 2007). One possible explanation is that AZT is damaging the mtDNA, whether through AZT incorporation into the DNA, disruptions of the dNTP pools, or a different mechanism. This damage may include fragmented mtDNA or mtDNA with deletions or mutations in it. The cell may try to up-regulate mitochondrial function by increasing mtDNA replication. Normally, more mtDNA would
allow more of the mitochondrial protein components of the electron transport chain to be made. However, much of the mtDNA present may be damaged and lead to the production of ineffective proteins if any proteins can be made at all. Thus even though there is more abundant mtDNA, the cell is still unable to function at full capacity. Longer continual exposure to AZT could result eventually in depletion as the damaged and fragmented mtDNA is degraded.

The data measuring the dNTP content of the 3T3-F442a cells could provide powerful evidence to support the alternative mechanism for AZT toxicity. Unfortunately, our data do not suggest that either AZT or d4T has an effect on the balance of the dNTP pools compared to the control. Even though d4T has never been shown to inhibit any of the deoxynucleoside salvage pathways, most of the few points that do show a statistically significant difference from the untreated control are associated with 10 µM d4T. Between Days 4 though 8, 10 µM d4T is associated with an increase in the percentage TTP. This is the only trend that varies from the control observed in the data. The remaining points are scattered with no meaningful pattern. Due to the lack of serum in the media, some changes are seen on Day 12. With few deoxynucleosides available for salvage, this results in some changes in dNTP pool
composition. However, none of the changes reflect depletion of TTP that could be attributable to AZT inhibition. Additionally, the lack of other molecules provided in the serum, such as growth factors, may play a role in initiating the variations observed on Day 12.

While these results do not provide any evidence to advance the proposed mechanism of AZT toxicity, they also do not disprove it. The protocol used could be modified to change the conditions to which the cells are exposed or when the samples are taken. Under the current protocol, the NRTI is added throughout the entire process of differentiation, including the portions where the cells are still actively replicating. While replicating, a process may occur that selects against cells that are most detrimentally inhibited by AZT, creating a population composed of cells immune to the effects of AZT. Instead the cells could be grown and differentiated completely before any NRTI is added. Once they are fully adipocytes on Day 6, NRTI could be added to the media and the cells maintained for several days. The differentiated cells would not be replicating, and the possibility for selection to occur would be reduced substantially. This could produce different effects on the dNTP pools.

A variation of the above modification could also be coupled with taking samples at shorter time points such as a few hours after the NRTI
is added. This could be done through the differentiation procedure and on differentiated cells. Longer exposure time would allow the cells to react to an early imbalance in the dNTP pools by changing gene expression. This expression change may result in the pools appearing normal after a few days, as was observed. This mechanism would be initiated by AZT affecting the dNTP pools, but the toxic effects would be caused by changes in the cell’s gene expression to compensate for the dNTP imbalance.

Another possible explanation of why no effect was observed in the current study is the Crabtree effect (Crabtree 1929). Cells grown in a high concentration of glucose use glycolysis even when oxygen is present, suggesting that the high glucose blocks oxidative phosphorylation. The Crabtree effect has been noted in a variety of immortalized cell lines, including, HepG2, HeLa, and AS-30D hepatoma (Marroquin et al. 2007; Rodriguez-Enriquez et al. 2001; Rossignol et al. 2004). These cells all have fully functional mitochondria but will use glycolysis when grown in high glucose (typically 25 mM in most commercially available media). When the cells are grown in media with galactose and no glucose, oxidative phosphorylation is used to generate ATP. Interestingly, this has a major effect on the toxicities of a variety of drugs known to inhibit
mitochondrial function. HepG2 cells were treated with rotenone, antimycin, oligomycin, and FCCP (Marroquin et al. 2007). When grown in 25 mM glucose media, the drugs had little effect or only an effect at a high concentration (FCCP). However, in the glucose-free 10 mM galactose media, all the drugs had a strong effect at a low concentration. It is possible that the high glucose media used in this study may be causing a Crabtree effect to occur in the 3T3-F442a cells. Thus if these trials were conducted in low glucose or glucose-free media, the NRTIs may have different effects due to the utilization of the mitochondria for maintaining the cellular ATP. Care must be taken to ensure that this media change would not adversely affect the cells growth or differentiation.

AZT competitively inhibits thymidine phosphorylation in isolated rat heart and liver mitochondria. Based upon this, we have proposed a mechanism for AZT toxicity. AZT inhibition of thymidine phosphorylation could deplete the TTP pool. This imbalance in TTP compared to the other dNTPs may result in mtDNA deletions and depletion, like that seen in tissues experiencing the adverse effects of AZT. However, this study has shown that AZT has little effect on the dNTP pools of 3T3-F442a cells and causes an increase, rather than the expected decrease, in mtDNA
content of these cells. These findings are contrary to the proposed mechanism. Nonetheless, further work must be done in order to determine what, if any, role inhibition of thymidine phosphorylation plays in generating AZT toxicity.
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