Towards an understanding of the side effects of anti-HIV drugs using Caenorhabditis elegans
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Chapter 7

General discussion
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Treatment with antiretroviral drugs has significantly improved the life expectancy of HIV-1 infected patients. However, exposure to antiretrovirals has been shown to cause a myriad of adverse events. Mechanisms behind drug toxicity, however, remain difficult to formulate and interpret when only patient data is considered. Most patients receiving antiretroviral medication do so in regimen form (HAART), or/and have switched regimes before, which hinders relating clinical readouts to the effect of a single drug. Complicating matters further, many patients fortify their diet with vitamin and mineral supplements in an attempt to bolster health and postpone adverse events. Nonetheless, advances have been made using in vivo and in vitro approaches. Unfortunately, such methods respectively pose limitations on the experiments that can be performed or limit their physiological relevance for whole organisms. Therefore progress in this field is highly dependent on robust and reliable integrated model systems.

In this thesis we have implemented and verified Caenorhabditis elegans as a suitable model system to study antiretroviral induced toxicity. Firstly we have discovered that NRTIs, which structurally are very similar, each have their own distinct mode of action and toxicity profile (Chapter 2-4). Secondly, our findings support the theory proposed by Apostolova et al.; that there are modes to NRTI toxicity beyond the scope of the polymerase-\(\gamma\) theory (Chapter 2-4). Thirdly, we have discovered that NRTIs and PIs can have rapid effects on mitochondrial function. Very little research has been done on the short-term effects of antiretrovirals and we show that these abrupt events need to be considered seriously as they have great impact on the manifestation of adverse events later on (Chapter 3-6). Finally, we have proven that C. elegans is an excellent model system to quickly screen combinations of antiretroviral drugs and in this way can expedite the discovery of beneficial or adverse drug interactions (Chapter 6).

1. NRTIs

1.1 Mitochondrial toxicity and the polymerase-\(\gamma\) theory

It has long been understood that the NRTI class of antiretroviral drugs causes severe adverse events in HIV-1 patients. Mitochondrial toxicity has been assigned as a common pathway underlying these adverse events. More specifically, the polymerase-\(\gamma\) theory has been proposed as a mechanism causative of mitochondrial toxicity.

Various steps in the polymerase-\(\gamma\) theory have been substantiated. NRTIs are known to compete with endogenous nucleotides and nucleosides for transcriptase binding. Due to their surplus and high affinity for polymerase-\(\gamma\), NRTIs are frequently incorporated into the new DNA strand which results in chain termination, as NRTIs all lack the 3'-hydroxyl group on the deoxyribose moiety necessary for phosphodiester bond formation in DNA strand elongation. Additionally, NRTIs have been proposed to inhibit the base-pair excision and proof-reading capacity of polymerase-\(\gamma\). Together this results in a reduced number of mtDNA molecules and eventually a reduction in mtDNA encoded proteins, which are essential components of MRC complexes.
1.2. Questioning the polymerase-\(\gamma\) theory

Besides decreased expression of MRC proteins, disruption of the MRC has also been assessed through evaluation of conventional mitochondrial functions. Membrane potential, oxygen consumption and ATP production have all shown negative alteration in the presence of NRTIs \(^{52,58,188}\). According to the polymerase-\(\gamma\) theory, these changes are the result of altered MRC protein expression and therefore diminished MRC function. However, studies have shown that not every case of mtDNA depletion by NRTIs leads to changed expression levels of mitochondrial respiratory chain proteins \(^{49,55}\). Similar observations were made in knockout mice deficient of the mtDNA transcription factor \(Tfam\), which showed reduced mtDNA content but normal levels of mitochondrial transcripts and mtDNA encoded respiratory chain subunits \(^{56}\).

Conversely, altered mitochondrial gene transcription and impaired respiratory chain activity have been observed in the absence of mtDNA depletion during NRTI exposure \(^{57,58}\). For instance, MRC complex activity has been shown to decrease significantly in the absence of mtDNA or polypeptide synthesis depletion \(^{51}\), and expression profiles of mitochondrial mRNA have been shown to adjust, both in a human cell lines and mice upon exposure to NRTIs \(^{57,60,63}\). These adjustments likely reflect cellular adaptation to pressure on the mitochondrial transcriptional machinery. Indeed, polymerase-\(\gamma\) deficient nematodes compensate for the decrease in mtDNA replication by up-regulating mitochondrial transcripts \(^{51}\). Furthermore, primate cell lines have been shown to adapt to inhibition of mtDNA gene expression by increasing the stability of mtDNA-encoded transcripts and proteins \(^{62}\).

1.3 A compensatory mechanism in \(C.\) elegans

In \(C.\) elegans we observed fluctuations in mtDNA copy number during exposure to NRTIs and, in light of the above, proposed that a compensatory mechanism may be at work to circumvent NRTI induced inhibition of polymerase-\(\gamma\) and subsequent mtDNA replication stalling (Chapter 2 & 3). In an attempt to unravel the compensatory response of \(C.\) elegans to NRTI thymidine analogue exposure we analysed mtDNA transcript quantity and mtDNA maintenance and transcription machinery transcript quantity using RNAseq. Although mtDNA copy number was lowered by d4T and FLT, transcripts generally remained unaltered (Chapter 4). This indicates that increased expression of maintenance and transcription machinery components is likely not the mechanism that \(C.\) elegans uses to compensate for mtDNA copy number decline.

It is possible that the response to NRTIs is \(C.\) elegans specific. \(C.\) elegans has a surprisingly low amount of recorded mtDNA mutations, whereas in humans over 100 point mutations and 200 insertions, deletions, or rearrangements have been described. It has been suggested that \(C.\) elegans is particularly impervious to mtDNA mutations either because of its tolerability to mutations or its ability to prevent their transmission \(^{189}\). A similar mechanism may underlie the ability of \(C.\) elegans to survive without polymerase-\(\gamma\) and sustain mtDNA encoded transcript quantity in the face of NRTI exposure. Further research into the roles of the genes related to ‘genetic information processing’ that were discovered in Chapter 4 may point towards such a mechanism.
Alternative mechanisms to compensate for mtDNA copy number decline may also include adaptations in mitochondrial morphology so as to evenly distribute mtDNA transcripts between organelles \(^{226}\), increased stability of mitochondrial mRNA or the storing of mRNA in so-called P-bodies \(^{389}\), or improved stability of mtDNA encoded proteins \(^{62}\). We have observed changes in mitochondrial morphology that coincide with the decrease in mtDNA copy number, indicating that this is a likely compensatory mechanism (Chapter 2). However, increased stability of mRNA and mtDNA encoded proteins are interesting candidates and warrant further investigation. It is also possible that heteroplasmy accounts for the lack of change in mtDNA encoded transcripts during exposure to NRTIs, as the amount of intact mtDNA copies, however few, may be sufficient to produce adequate amounts of transcripts \(^{345}\). This idea is supported by the observations of Bratic et al. who demonstrated that polymerase-\(\gamma\) deficient nematodes were able to reach adulthood in relatively good health despite low copies of mtDNA. Polymerase-\(\gamma\) deficient nematodes, however, are either sterile or die prematurely \(^{61}\), indicating that any survival adaptations to polymerase-\(\gamma\) inhibition or a reduced mtDNA copy number by NRTIs are likely to prove successful for only short periods of time. Continued repression of mtDNA transcription for longer periods will eventually result in MRC failure due to the perturbed turnover of mtDNA encoded transcripts and MRC proteins. This is likely especially the case, when under constant inhibition, polymerase-\(\gamma\) generates truncated mtDNA copies or during transcription can no longer reach the end of its templates, creating non-polycistronic transcripts \(^{65}\). Furthermore, these components remain susceptible to ROS induced damage that increases upon deterioration of MRC function, a perpetual cycle of increased ROS generation more commonly known as the vicious cycle of ROS in the mitochondria \(^{165}\).

### 1.4 Toxicity mechanisms besides the polymerase-\(\gamma\) theory

Proposed modes of toxicity beyond the polymerase-\(\gamma\) theory include; NRTI metabolism and pharmacokinetics, direct interaction of NRTIs with mitochondrial components besides polymerase-\(\gamma\), and the generation of ROS and ROS mediated signalling \(^{32}\). In this thesis we have attempted to approach each of these themes and have found that they all play important roles in the development of adverse events.

#### 1.4.1 Direct inhibition of MRC components

We discovered that within minutes after NRTI exposure, ATP and oxygen consumption levels were reduced, indicating direct inhibition of MRC function (Chapter 3). We also observed that whilst mtDNA replication was also rapidly stalled, these events occurred too quick for them to be a consequence of polymerase-\(\gamma\) inhibition and consequent MRC failure as described in the polymerase-\(\gamma\) theory. Direct inhibition of MRC components, in particular of complex I, has been observed for AZT and ddC \(^{68-70}\). We, however, propose that all first-generation NRTIs are able to directly inhibit MRC function and cause a cascade of events that at least partially underlie the development of adverse side effects during chronic exposure (Chapter 3). On the basis of structural features alone, NRTIs likely effectively compete with the nicotinamide adenine dinucleotide (NAD) recognition site of Complex I \(^{68}\). Complex I has many suspected nucleotide binding sites \(^{69}\), making it the most likely of MRC complexes to interact with NRTIs. The similarity of NRTI induced adverse events to symptoms observed in inherited mitochondrial disorders that specifically affect NADH binding sites, such as the early onset of neurodegenerative disorders, lactic acidosis, cardiomyopathy, and exercise intolerance, also supports this.
Moreover, NRTI exposure and mutations in the Complex I NADH binding sites have both been shown to increase ROS production.

### 1.4.2 Increased ROS generation and mitohormesis

One major consequence of rapid inhibition of the MRC and chronic mitochondrial toxicity is the increased generation of mitochondrially derived ROS. Besides the undoubted detrimental effects of oxidative stress such as the damage of proteins, lipids and DNA which likely induce and aggravate adverse events, we propose that NRTI induced ROS production also triggers signalling pathways that affect fitness and longevity. Specifically, MRC inhibition and ROS production induce mitohormetic signalling pathways similar to those seen in mit mutants (Chapter 3 & 4). ROS are known to dictate biological outcomes and their precise influence has been suggested to regulate different biological outcomes. The consequence of ROS generation has been found to depend its concentration, as proposed in the “gradual ROS response” theory.

A mitochondrial ROS production level that exceeds that which can be quenched by the innate anti-oxidant system can be interpreted as a stress signal. Finkel et al. proposed that the intensity or duration of such a fluctuation in ROS determines the biological outcome through redox-dependent signal transduction. For example, metabolic adaptations which take place during temporary hypoxia or with changes in glucose metabolism are likely triggered by low amounts of ROS. Moderate levels of ROS may trigger inflammatory mediators, and finally, high levels of ROS can induce autophagy or apoptosis pathways incurring cell death.

In *C. elegans*, DEGs regulated during exposure to AZT, d4T, and FLT, showed an approximate 10% overlap with genes that were regulated in the mit mutants *isp-1* and *nuo-6* (Chapter 4). Mitochondria from both mit mutants have been showed to produce elevated levels of ROS. This relatively small overlap in genes between NRTIs and mit mutants does not necessarily mean that the NRTIs do inhibit MRC function. On the contrary, RNAi knock-down of MRC complex components that are identical to mit mutant deficiencies, have been shown to have distinctly different metabolic profiles.

Additionally, AZT showed an approximate 20% overlap with down-regulated genes and a 56% overlap with up-regulated genes induced by exposure to 100μM paraquat, which is known to induce mitohormetic signalling through an increase in ROS. Besides the known inhibition of MRC components by AZT, the azide group of AZT can rapidly induce both nitric oxide and superoxide anion production, and azide is known to strongly inhibit cytochrome c oxidase (complex IV) function, which together may also explain the large overlap in genes between AZT and paraquat. Nonetheless, impaired electron transport often increases oxidative stress which can lead to an increased expression of ROS defense mechanisms and eventually result in lifespan extension. Although our results in chapter 3 strongly suggest that NRTIs cause similar responses to MRC inhibition, we have not directly measured the extent of MRC inhibition or ROS generation.

To assess the production of *H₂O₂* in *C. elegans*, we used H₂DCFDA, which has known limitations (discussed in chapter 3). The observed decrease in global ROS production rate measured by H₂DCFDA correlates well with a
decrease in MRC function (Chapter 3). Due to the relatively long half-life of H$_2$O$_2$ above other ROS, H$_2$O$_2$ is considered to be an important signaling molecule and has been shown to mediate mitohormetic responses. However, the type of ROS produced and the compartmentalization of the generated ROS are important factors that regulate distinct biological outcomes. Application of H$_2$DCFDA in the timeframes described in chapter 3, predominantly reflects ROS levels in the mitochondrial inter-membrane space and the cytosol. ROS generated in the mitochondrial matrix are unlikely to be assessed using this approach, yet ROS generated in the mitochondrial matrix does have considerable impact on mitochondrial function and consequently mitohormetic signalling. Paraquat, for instance, is known to generate ROS in the mitochondrial matrix at the site of complex I. The inhibition of the MRC by NRTIs probably also results in generation of ROS within the mitochondrial matrix, which cannot readily be detected by H$_2$DCFDA. This notion is supported by the fact that MRC complexes predominantly generate ROS within the mitochondrial matrix, and we showed that all NRTIs altered quionone redox status (Chapter 2). A potential site for NRTI induced MRC inhibition and consequent ROS generation would therefore be complex I.

1.4.3 NRTI pharmacokinetics and inhibition of mitosis

We have observed that NRTI pharmacokinetics play a major role in the development of adverse events, in particular in rapidly replicating tissues (Chapter 4). Using RNAseq transcriptome analysis we discovered that the introduction of relatively high concentrations of thymidine analogues into replicating tissues can disrupt mitosis. We propose that this phenomenon is similar to the synchronisation method used in cell lines, more commonly referred to as ‘thymidine block’. Additionally, we observed that the expression of genes that encode enzymes that play a role in thymidine analogue transport, phosphorylation and detoxification, are altered upon exposure to thymidine analogues. Taken together, these results support the theory that nucleoside and nucleotide pool imbalance plays an important underlying role in NRTI toxicity.

The transport of nucleosides and nucleotides into mitochondria, or other subcellular compartments, has been proposed as a major determinant of mitochondrial effects of NRTIs. Studies of the role of such transporters may shed light upon specific drug effects and the susceptibility of specific tissues to NRTI toxicity. Further research is needed, however, to fully understand the consequences of thymidine analogue induced nucleoside and nucleotide pool imbalance, and elucidate this insufficiently investigated research area of NRTI toxicity.

This mode of toxicity for NRTI thymidine analogues also falls outside the scope of the polymerase-γ theory and we suggest that this is a major contributor to adverse events, particularly those that effect replicating tissues (Chapter 5).

2. PIs

2.1 Mitochondrial toxicity

PIs are a frequently prescribed drug class in antiretroviral therapy and besides the NRTIs they are the most widely used class in HAART. PIs competitively bind the viral aspartyl-protease active site with high affinity and therefore inhibit cleavage of viral polyproteins and subsequent maturation of the virion after budding from the host cell. In this way PIs hinder viral replication and consequent infection of other cells. In comparison to
NRTIs, little research has been done to categorize and elucidate PI toxicity. However, PI induced adverse events, much like adverse events caused by NRTIs, are similar to those observed in patients suffering from mitochondrial diseases, implying that PIs induce mitochondrial dysfunction \(^{33}\). In support of this, recent studies have demonstrated that PIs affect expression levels of mitochondrial encoded MRC subunits, mitochondrial membrane potential and mitochondrial morphology \(^{95-96}\). Importantly, PIs have also been shown to induce elevated mitochondrial ROS production, possibly via depolarization of mitochondrial membrane potential \(^{102}\) or decreasing Cu/ZnSOD gene expression \(^{99}\). The precise cause for PI induced adverse events, however, is - to date - unknown.

Reyskens and Essop proposed that PIs deplete ROS detoxification enzymes in the cytosol, raising ROS within the mitochondrial inter-membrane space and consequently inhibiting mitochondrial function \(^{99}\). In support of this, we found that PIs rapidly decrease GST-4 expression and diminish MRC function. MRC function could be attenuated by AOX which strongly suggests that mitochondrial dysfunction is caused by an increased generation of ROS (Chapter 5). As with the NRTIs, however, the origin and localization of the produced ROS remains elusive. To observe if ROS are produced within the mitochondrial matrix, we suggest the use of MitoSOX as it is superoxide specific and rapidly localizes in the mitochondria. Moreover, mitochondrial targeted AOX such as MitoVit-E, MitoQ, or MitoTEMPO could be implemented \(^{426,437}\).

### 2.2 Other modes to PI induced adverse events

Although a direct mechanism behind PI induced mitochondrial dysfunction is gradually appearing, PIs have been proposed to inhibit many other cellular functions. For instance, PIs have been found to inhibit proteasomal degradation of newly synthesized apolipoprotein B, the principal structural component of triglyceride and cholesterol-rich plasma lipoproteins \(^{116}\). Additionally, PIs have been suggested to inhibit lipoprotein lipase \(^{115}\), cytoplasmic retinoic-acid binding protein type 1 \(^{115}\), the mitochondrial processing protease \(^{118}\), and the integral membrane zinc metalloprotease ZMPSTE24 \(^{95,120}\). Further research into these potential modes of PI toxicity is warranted, although the use of \textit{C. elegans} herein remains to be revealed. One fascinating area of PI research in which \textit{C. elegans} may be useful is the modified activity and expression of active drug transport systems \(^{374}\). Such changes may alter drug absorption, elimination, tissue distribution, and ultimately the occurrence of adverse events. RTV, for instance, is known to have the strongest inhibitory effect on drug transport systems, and is therefore used to ‘boost’ HAART by increasing bioavailability and half-life of concomitantly administered drugs \(^{27}\). Further research into the ability of PIs to change antiretroviral pharmacokinetics may help the use of PIs in combination therapy (see Chapter 6).

### 3. \textit{C. elegans} as a model system

There are multiple \textit{in vivo} and \textit{in vitro} model systems in use to study drug toxicity (Chapter 1). However, their complexity and often divergence from a realistic reflection of \textit{in vivo} situations hampers their contribution to this field. The lack of a good model system has hindered consistent and coherent research into discovering adverse effects of antiretroviral therapy \(^{52}\).
C. elegans has several advantages over other model systems as it is multicellular, highly malleable and transparent. C. elegans normally has a relatively short lifespan of two weeks, enabling researchers to rapidly assess the effects of different mutations or treatments on lifespan. Although limited, this model system can help researchers dissect tissue- and compartment-specific effects, as C. elegans has highly differentiated tissues including, neuronal, gonad, intestine, muscle, and cuticle tissue. Additionally, C. elegans has the advantage that its genome is fully sequenced and RNA interference is relatively simple, enabling targeted gene knock-down.

The nematode Caenorhabditis elegans has proven itself to be one of the most versatile model organisms for the elucidation of molecular pathways implicated in many human diseases, including those of mitochondria and ageing. Ageing in C. elegans, for instance, is entirely post-mitotic, reflecting the gradual loss of function in somatic cells as they grow old. This nematode has also specially been used to study drug-specific impact on mitochondria. Mitochondrial research in C. elegans has given us many insights into the genetic regulation of ageing and mitochondrial function, and it has provided us with a vast array of mutants to study these effects. With this knowledge we can use C. elegans to quickly evaluate the effects of antiretroviral drugs, not only on mitochondrial function directly, but in relation to organism genetics, physiology and longevity.

3.1 C. elegans mitochondria

As adverse effects of antiretrovirals have, first and foremost, been proposed to stem from mitochondrial dysfunction, the homology between C. elegans and human mitochondria is important to address. The C. elegans mitochondrial genome is 13.794 nucleotides in length and is therefore slightly smaller than its human counterpart. The C. elegans mtDNA encodes 36 genes: 2 ribosomal RNAs (12S and 16S rRNA), 2 transfer RNAs, and 12 subunits of the MRC. It differs in a few aspects from the human mtDNA as it misses the ATP8 gene which encodes a subunit of the ATP synthase (Complex V), and the arrangement of genes on C. elegans mtDNA differs from that in humans.

As polymerase-β is necessary for mtDNA replication and NRTIs are known to inhibit polymerase-β, it is also important to address the similarity between C. elegans and human polymerase-β. The C. elegans polg-1 (Y57A10A.15) encoded amino acid sequence has a 26% identity and 45% similarity to the human POLG and harbours highly conserved exonuclease and polymerase domains. C. elegans polg-1 deletion mutants have reduced mtDNA copy number and mtDNA encoded transcript quantity, indicating that POLG-1 is indeed necessary for mtDNA replications similar to humans. The highly conserved domains and the observations that some NRTIs could deplete mtDNA copy number in C. elegans strongly suggests that C. elegans is a suitable model system to study NRTI induced mtDNA replication defects.

Bratic et al. discovered that mtDNA replication in C. elegans predominantly occurs in the proliferating gonads at late larval and early adult stages. Specifically, mtDNA copy number rises sharply from approximately 250,000 in the L4 stage to 600,000 in D1 adults. We used this phenomenon to our advantage and by exposing L4 nematodes to antiretrovirals showed that they can rapidly induce fluctuations in mtDNA copy number.
Additionally, Bratic et al. demonstrated that mtDNA copy number is dependent on the temperature at which the animals are cultured. Temperatures above or below the typical 20°C showed increased mtDNA copy numbers, and at 25°C nematodes also showed a higher mtDNA turnover rate, indicating metabolic stress. In light of this, all our mtDNA copy number experiments were performed at 20°C.

### 4 Practical challenges

#### 4.1 DMSO

The antiretrovirals used in this thesis have been dissolved in dimethyl sulfoxide (DMSO), as is common for administering compounds to *C. elegans*. DMSO is an aprotic solvent that has been proposed to closely represent the ‘hydrophobic biological matrix’ of organelles, such as mitochondria. Nonetheless, DMSO is known to be a confounding factor, and use of DMSO in *C. elegans* has also been shown to increase cuticle permeability and influence the uptake of administered compounds.

For *C. elegans* the confounding effects of DMSO are dependent on the experiment being performed and the DMSO concentration in use. For instance, on solid NGM, DMSO showed a biphasic dose response in lifespan assays; at low concentrations (≤0.2%, v/v) DMSO showed no effect on lifespan, moderate concentrations (0.5%-2%) extended mean and maximal lifespan, whereas high concentrations (≥5%) shortened mean and maximal lifespan. On the other hand, 0.1% DMSO has been shown to have no confounding effects on *in vitro* measurements of the superoxide scavenging potential of selected compounds, and concentrations below 1% had no effect on progeny number in *C. elegans*.

For the administration of NRTIs to *C. elegans* cultured on solid NGM, the final concentrations (v/v) of DMSO were 0.067% (AZT, d4T, ddI & FLT) and 0.2% (ddC). We exposed nematodes to PIs with a final concentration of 0.3% (IDV & NFV) and 3% (RTV & SQV) DMSO. With the PIs we observed that they were often difficult to dissolve in M9 buffer or NGM. Similarly, this was also observed by Mukhopadhyay *et al.* who needed a final concentration of ≥15% DMSO to solute the PIs IDV, RTV, and SQV. Conversely, Caron *et al.* were able to use IDV and NFV with final concentrations of DMSO ≤0.005%, and Lagathu *et al.* used controls of 0.1% for IDV, NFV, and RTV. To circumvent the problems of PI solubility in M9 buffer or dH2O, for instance in the plate reader experiments using GST-4 expression and H2DCFDA, we used citrate phosphate buffer (pH=6.5).

Taken together, we have strived to keep DMSO levels to a minimum and where possible we have included relevant DMSO controls. However, we cannot entirely exclude the fact that DMSO may have confounding effects on some experimental outcomes. This may be of particular relevance in the case of longevity studies, as the lifespan extending effect of DMSO has been found to depend on the DAF-16/forkhead transcription factor and the NAD-dependent histone deacetylase-like protein SIR-2.1, which can mediate many longevity effects.

#### 4.2 FUdR

5-fluoro-2’-deoxyuridine (FUdR) is frequently used with *C. elegans* to maintain synchronous cultures. To this effect we have also used FUdR in experiments that overlapped with the nematodes reproductive phase, for instance during longevity and fitness trials. A recent study assessed the effects of FUdR on *C. elegans*.
mitochondrial function and morphology, and mtDNA and nDNA copy numbers. Mitochondrial ATP levels and mitochondrial morphology remained unchanged at low (25μM) and high (400μM) concentrations. MtDNA and nDNA levels decreased slightly upon exposure to FUdR, but their ratios were not altered. The concentration of FUdR, however, has been shown to affect nematode lifespan. Concentrations of approximately 400μM have lifespan extending effects, and it has been suggested that this is mediated by improved proteostasis. We therefore used a final concentration of 50μM FUdR throughout this thesis and do not expect the presence of FUdR to have lifespan extending effects.

Perhaps of more concern to us, however, is that FUdR induces sterility through inhibition of thymidylate synthase. This results in an imbalance of deoxyribonucleoside triphosphate pools causing the accumulation of DNA double strand breaks and thus impaired cell division of the nematodes germline stem cells. In chapter 4 we proposed that introduction of thymidine analogues, specifically d4T and FLT, causes imbalances in nucleoside pools leading to defective mitosis and decreased progeny number. FUdR is also structurally similar to endogenous nucleosides and NRTIs, and FUdR has been found to act as a substrate for thymidine kinase, much like NRTIs. It is therefore probable that FUdR interferes with NRTI pharmacokinetics and vice versa. Phosphorylated forms of d4T for instance have been found to increase upon co-incubation with FUdR in a human T-lymphoblast cell line. In addition, we have observed that exposure to AZT diminished the ability of FUdR to induce sterility, resulting in a few viable progeny (data not shown), which is in line with a previous report that showed ‘antioxidant’ plant extracts occasionally caused larvae to escape the action of FUdR at low concentrations (≤50μM). We observed that AZT induced a similar gene expression profile to 100μM paraquat (Chapter 4), which may indicate that the oxidative stress response induced by AZT circumvents the effect of FUdR.

4.3 Drug concentrations

In HIV-1 patients, the plasma concentration of the antiretroviral drugs used in this thesis is generally between 4-16µmol/L. However, plasma concentrations of 50µM have been observed for AZT in the clinic. In vitro experiments use very low concentrations (pM) up to approximately 90 mM, although concentrations between 10-200μM are most common. We initially selected a concentration range of 100-200μM for NRTIs on the basis of FLT’s ability to deplete mtDNA copy number (Chapter 2) and selected 100-300μM for PIs based on results from R. de Boer (data not shown). Moreover, these selected concentrations for all antiretrovirals showed robust effects on mitochondrial function (Chapter 3 & 5).

Drugs can be exposed to nematodes on solid media, by either mixing them in with the bacterial lawn or adding them directly to the agar plates. We found both methods to be effective, yet preferred mixing the drugs into the agar plates so as to limit drug diffusion over time. In this way, there are two ways in which the antiretrovirals can reach their targets, namely through ingestion or by diffusion across the nematodes cuticle. The cuticle of C. elegans is notoriously impervious to many compounds and drug uptake by C. elegans is rather poor. Moreover, C. elegans has extensive enzymatic xenobiotic defences and exogenously applied pharmacologicals that do penetrate the cuticle often fail to accumulate to effective concentrations within...
tissues. It is not therefore uncommon for polar drugs to be applied in a concentration 1000 fold higher than their predicted affinity for the target.

Although the drug concentrations used in this thesis have been chosen with care, the ability of NRTIs to induce adverse events is known to be dependent on the NRTIs phosphorylation state. The detection of NRTI-TPs, however, in patients is difficult and has limited pharmacological research into the pharmacokinetics of NRTIs. Measurements of NRTI nucleoside and nucleotide concentrations within nematodes may help explain discrepancies between the toxicity profiles of each NRTI and shed light on the pharmacokinetics of each compound.

4.4 Drug delivery

With the exception of quinone redox state and RNAi experiments, C. elegans was cultured on live monoxenic E. coli OP50 cultures throughout this thesis. For the administration of compounds to C. elegans both live and dead E. coli can be used, although UV radiation killed E. coli are preferred so as to circumvent possible drug metabolism by E. coli. Using UV killed E. coli, however, has been shown to be very stressful for C. elegans. Specifically, nematodes raised on UV killed E. coli have a reduced number of progeny, slowed ageing and an increased lifespan, suggesting that diet-stress heightens oxidative stress and induces hormetic effects. This is supported by the discovery that the hormetic effects of chronic paraquat exposure were only observed when nematodes were fed live E. coli. In light of this, and because the studies in this thesis focus on stress and mitohormetic responses as a result of antiretroviral drug exposure, we chose to use live E. coli.

We showed that for nematodes treated with AZT, mtDNA copy number did not differ between live or UV killed E. coli. We did this to demonstrate that the growth inhibitory effect of AZT did not affect nematode physiology or drug properties (Chapter 2). It is still possible, however, that live E. coli metabolize and phosphorylate the NRTIs. C. elegans possesses a cytoplasmic kinase (THK-1) that displays a high activity for thymidine but low or no activity with deoxyguanosine, deoxycytidine, and deoxyadenosine (Chapter 2). Therefore, the phosphorylation of thymidine analogues, like AZT, will take place regardless of a live or dead food source. For ddC and ddI, however, it is unclear if they will become phosphorylated by C. elegans. C. elegans has been found to encode a deoxycytidine kinase protein homologous peptide (C14B9.2), yet its function remains putative. On the other hand, enzymes are present in E. coli that can phosphorylate cytidine to cytidine-TP. However, exogenously supplied cytidine does not flow through this pathway. Instead, a very active cytidine deaminase rapidly converts exogenously supplied cytidine to uridine, which then flows through a portion of the de novo pyrimidine biosynthesis pathway to uridine-TP and then to cytidine-TP. The affinity of this pathway for NRTIs, however, is unknown.

The depletion of mtDNA observed in chapter 2 and 3 upon exposure of ddC or ddl, however, suggests that either E. coli or C. elegans phosphorylates these analogues, or in addition that their effects are non-specific to polymerase- inhibition and may involve interference with other steps in nucleotide metabolism such as nucleoside pool imbalance. More research is therefore warranted to see if the effects of ddC and ddl are unrelated to their phosphorylation state and will shed some light on the toxicity of NRTIs beyond the.
polymerase-\(\gamma\) theory. Preliminary microarray genome wide expression data of nematodes exposed to ddC for 24h on live *E. coli* (data not shown) indicated that ddC induced processes that were similar those induced by AZT, d4T and FLT in chapter 4, namely cell division, the spliceosome, proteostasis, and metabolism of nucleobases, lipids, and nitrogen. Additionally, ddC exposure induced genes expression was directionally almost completely opposite to those expressed in the mit mutant *gas-1*.

5. Concluding remarks
Many questions remain unanswered in the antiretroviral drug field (See Chapter 1). Taken together, this project has endeavoured to clarify the short-term effects of antiretroviral drugs in order to understand the mechanisms behind their toxicity. In particular, we have focused upon mitochondrial dysfunction and have attempted to broaden the scope of research upon antiretroviral induced adverse events by addressing modes of toxicity beyond the polymerase-\(\gamma\) theory. We hope that this thesis will help elucidate the effects of HIV-1 medicines and not only shed some light on their modes of toxicity, but will also benefit the development of new, effective, and less toxic compounds.