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

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Chapter 6

Analysis of Nucleoside Reverse Transcription Inhibitor and Protease Inhibitor combination therapy in *C. elegans*
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Abstract

It is well known that HIV-1 antiretroviral drugs can cause severe adverse events; however, the elucidation of the mechanisms underlying their toxicity is hampered due to the fact that antiretroviral drugs are prescribed in complex combinations. Drug interactions can alter the absorption, distribution, metabolism, or excretion of administered compounds. Additionally, the pharmacologic actions of each drug can be altered through additive, synergistic, or antagonistic effects, which collectively can result in altered drug bioavailability. Combinations of antiretrovirals are commonly referred to as highly active antiretroviral therapy (HAART) and despite their toxicity, HAART use has significantly reduced HIV-1 related mortality. Most HAART regimens include the two major antiretroviral drug classes; nucleoside reverse transcription inhibitors (NRTIs) and protease inhibitors (PIs). In this study we use Caenorhabditis elegans and verified techniques to investigate the potential of first-generation NRTI and PI combinations to induce mitochondrial dysfunction. We discovered that combinations of FLT + NFV, ddI + RTV, and d4T + SQV had the highest potential to generate mitochondrial toxicity. Combinations of AZT + NFV, RTV, or SQV, combinations of d4T + IDV or NFV, and combinations of ddC + NFV or SQV, had the lowest potential to generate mitochondrial toxicity. This study further supports the use of *C. elegans* for high-throughput screening of multiple complex regimens to assist in diminishing the prescription of drug combinations that unfavourably interact with each other and have strong potential to induce adverse side effects.

1. Introduction

In recent years it has become increasingly clear that treatment with therapeutic drugs can result in severe side effects [10, 102]. One important disease where this has clearly been demonstrated is HIV-1 infection, with over 50% of the patients developing severe side effects that force them to change their therapeutic regimen [32]. The nucleoside reverse transcriptase inhibitors (NRTIs) were the first class of drugs approved for treatment of HIV-infected individuals, and with great success, as HIV-1 related fatality decreased rapidly [9]. Soon after, the protease inhibitor class (PIs) was introduced, opening the era of Highly Active Antiretroviral Therapy (HAART). HAART guidelines consist of a combination of two NRTIs combined with a Protease Inhibitor (PI) or a Non-Nucleoside Reverse Transcriptase Inhibitor [14]. Since the introduction of HAART, at least in high-income
countries, HIV-1 infection has acquired the characteristics of a chronic disease; manageable yet requiring life-long treatment.

1.1 NRTIs

For a major early step in the HIV-1 replicative cycle, the viral reverse transcriptase converts its viral RNA into viral DNA. NRTIs make use of this process by inhibiting the viral reverse transcriptase as DNA incorporation with NRTIs results in chain termination. Specifically, all NRTIs lack the 3’-hydroxyl group on their ribose moiety necessary for phosphodiester bond formation in viral DNA strand elongation. Human mitochondrial DNA (mtDNA) polymerase-γ, however, also has high affinity for NRTIs. Inhibition of polymerase-γ by NRTIs can interfere with mtDNA replication and can cause mitochondrial toxicity (Chapter 1). NRTI induced mitochondrial toxicity is, therefore, commonly referred to as the “polymerase-γ theory”. Dysfunction of the mitochondria seems to be the underlying cause for NRTI related adverse effects, which can range from mild, like myopathy, to fatal, such as pancreatitis, liver failure and lactic acidosis (Chapter 1).

1.2 PIs

In contrast to the NRTIs, PIs target later stages in the HIV replication cycle. Following the integration of the HIV-1 reverse transcribed proviral DNA into the host cell’s DNA, viral RNA is translated into viral polypeptides. These polypeptides need to be cleaved by the virally encoded protease to form functional proteins and infective virions (Chapter 1). PIs function through competitive inhibition of the viral protease so as to prevent the production of mature virions. PIs use is known to be related to several adverse effects such as hyperglycaemia, lipodystrophy, liver failure and hypersensitivity syndrome (Chapter 1). Although their exact mode of toxicity is unknown, the observed mitochondrial effects of PIs are typically related to an increase in reactive oxygen species (ROS) production, changes in mitochondrial membrane potential (ΔΨ\textsubscript{m}), a decrease in ATP generation, and the initiation of apoptotic events (Chapter 5).

1.3 HAART

HAART utilizes the combination of NRTIs and PIs to increase therapy efficacy, overcome problems of tolerance, and decrease emergence of viral resistance. However, interactions between NRTIs and PIs have been reported, and the toxicity and concurrent development of adverse events varies greatly per administered combination. Combinations with zalcitabine (ddC), for instance, have been found to cause a high incidence of peripheral neuropathy. On the other hand, combinations of indinavir (IDV) or nelfinavir (NFV) with either zidovudine (AZT), stavudine (d4T), or didanosine (ddI), and saquinavir (SQV) with either AZT or ddI, are still advised for therapy of antiretroviral naive adult patients despite the implementation of newer and less toxic drugs.

Finding a good therapy regimen is highly dependent on the patient in question, and the discovery and avoidance of contraindications in the clinic has often relied on tentation. Patients can react very differently to standardized formulations which can force them to switch their regimen. Reasons for regimen switching include the occurrence of adverse events, co-morbidities such as hepatitis, or viral rebound because of acquired drug resistance. Surprisingly, very little \textit{in vitro} or \textit{in vivo} research has been done in an
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attempt to discover particularly pernicious combinations. Combinations of IDV with AZT have been tested for toxicity in cell lines and research has shown that these combinations can induce an increase in mitochondrially derived ROS, cause loss of ΔΨ\textsubscript{mt}, and in some cases instigate apoptosis.

1.4 Aim of this study
In previous chapters of this thesis we have established C. elegans as a suitable in vivo model system to study antiretroviral drug induced toxicity and we have investigated modes of action behind NRTI and PI induced mitochondrial dysfunction (Chapter 2-5). In this study we explore the adverse events related to mitochondrial toxicity from first-generation NRTI and PI combinations, in an attempt to gain insight into the additive, synergistic or antagonistic effects these drugs can have on mitochondrial function.

2. Results
2.1 mtDNA copy number
In our previous work using quantitative PCR we determined that exposure to some NRTIs can lead to severe mtDNA depletion. We also observed that PIs can cause fluctuations in mtDNA copy number (Chapter 5). In light of this we set out to measure mtDNA copy number in worms exposed to combinations of first generation PIs and NRTIs (Figure 1). Combinations of one NRTI (200μM) with one PI (100μM) showed that IDV + alovudine (FLT) drastically reduced mtDNA copy number compared to the control and FLT alone. IDV + ddC also decreased mtDNA copy number compared to the ddC alone (Figure 1A). NFV + FLT showed a marked decrease in mtDNA copy number compared to the control. NFV with either ddI or FLT decreased copy number compared to the NRTI alone (Figure 1B). Ritonavir (RTV) with ddI or FLT showed decreased mtDNA copy number compared to the control, and exacerbated the decline compared to the NRTI alone (Figure 1C). SQV in combination with any NRTI caused considerable mtDNA copy number decline compared to the control and compared to the NRTI alone (Figure 1D).

2.2 Mitochondrial morphology
Mitochondrial morphology is known to play essential roles in mtDNA rescue, protein quality control and cell survival. Previous studies in C. elegans demonstrated that singular exposure to NRTIs and PIs can cause significant alterations in mitochondrial morphology (Chapter 5). We therefore quantified mitochondrial morphology change using area, solidity, and entropy, as well-defined descriptors of mitochondrial fitness (Appendix). The solidity metric can be used to quantify the state of mitochondrial fusion and fission, and the entropy metric can be used to quantify the collective organisation of mitochondrial networks.
Figure 1: Relative quantities of mtDNA after exposure to combinations of NRTIs and PIs. NRTI concentration = 200μM, PI concentration = 100μM, Exposure time = 72h. Error bars represent 95% confidence intervals (16df). Significance was determined on the normalised CT values using a two-tailed student’s t-test assuming unequal variance. * = P-value <0.05, ** = P-value <0.01, *** = P-value <0.001.

Combinations of IDV with AZT, d4T, ddC, or FLT, increased mitochondrial area, whereas IDV + ddI showed no change compared to the control. IDV with either ddI or FLT also significantly increased the area compared to the NRTI alone (Figure 2A). IDV with AZT or ddI increased mitochondrial solidity compared to the control and compared to the NRTI alone, and IDV with d4T, ddC, or FLT, showed no change (Figure 2B). Mitochondrial entropy was increased for IDV + AZT compared to the control, and IDV + d4T showed a decrease in entropy compared to d4T alone (Figure 2C). Combinations of NFV with either ddC or FLT increased mitochondrial area, and NFV + ddI decreased mitochondrial area compared to the control. NFV + ddC and NFV + FLT also increased mitochondrial area compared to the NRTI alone (Figure 2D). Mitochondrial solidity was decreased with NFV + ddC compared to the control, and NFV + d4T increased solidity compared to d4T alone (Figure 2E). NFV with AZT or ddI increased mitochondrial entropy compared to the control, and NFV + d4T decreased entropy compared to d4T alone (Figure 2F). RTV combinations with AZT, ddC, or FLT, caused mitochondrial area to increase, and RTV + d4T decreased mitochondrial area compared to the control. RTV with either AZT or ddC also increased area compared to the NRTI alone (Figure 2G). Combinations of RTV with any NRTI showed no change in mitochondrial solidity compared to the control. Only RTV + FLT increased solidity compared to FLT alone (Figure 2H). RTV + ddI increased mitochondrial entropy compared to the control and ddI alone, and RTV + AZT decreased entropy compared to AZT alone (Figure 2I). SQV + ddC increased mitochondrial area compared to the control, and SQV + d4T decreased area compared to d4T alone (Figure 2J). Only SQV + AZT increased mitochondrial solidity compared to the control (Figure 2K). SQV with either AZT or ddI increased mitochondrial entropy compared to the control. SQV + d4T and SQV + ddI decreased and increased mitochondrial entropy respectively compared to the NRTI alone (Figure 2L).
Figure 2. Mitochondrial morphology analysis of NRTI and PI combinations. NRTI concentration = 100μM, PI concentration = 100μM, Exposure time = 72h. Boxplots comparing selected metrics (Area, Solidity, & Entropy) per condition, A-C: IDV + NRTIs; D-F: NFV + NRTIs; G-I: RTV + NRTIs; J-L: SQV + NRTIs. Statistics were calculated by two sided student’s t-test assuming unequal variance. * = P<0.05, ** = P<0.01, *** = P<0.001.
2.3 ATP levels

Besides the effects of NRTI and PI combinations on mitochondrial toxicity after prolonged exposure, immediate changes in mitochondrial function can also be highly informative (Chapter 3 & 5). The turnover of ATP in healthy, active mitochondria is high, which makes transient ATP levels a good marker for acute mitochondrial dysfunction. We previously showed that singular exposure to NRTIs or PIs rapidly reduced ATP levels in *C. elegans* (Chapter 3 & 5), so we next measured ATP levels during NRTI and PI combinations.

IDV with either d4T or ddI increased ATP levels, and IDV + FLT decreased ATP levels compared to the control. Additionally, IDV with AZT, d4T, ddC, or ddI, increased ATP levels compared to the NRTI alone (Figure 3A). NFV with either AZT, d4T, ddI, or FLT, decreased ATP levels, and NFV + ddC increased ATP levels compared to the control. Combinations of NFV with AZT, ddI, or FLT, also decreased ATP levels compared to the NRTI alone, whereas NFV + ddC increased ATP levels compared to ddC alone (Figure 3B). RTV in combination with any of the NRTIs caused decreased ATP levels compared to the control. RTV with d4T, ddC, or ddI also decreased ATP levels compared to the NRTI alone (Figure 3C). SQV in combination with any NRTI induced lower ATP levels compared to the control (Figure 3D).

Figure 3. ATP level analysis after exposure to NRTI and PI combinations. NRTI concentration = 200μM, PI concentration = 300μM. ATP levels were measured after 1:50 minutes of exposure. ATP statistics were calculated with a two-way ANOVA with replication. * = P<0.05, ** = P<0.01, *** = P<0.001. Error bars indicate standard error.

Low concentrations of RTV are often used in HAART as a ‘booster’, as RTV has been shown to be an effective inhibitor of CYP3A function. CYP3A enzymes actively metabolize drugs and by inhibiting their activity RTV can increase drug bioavailability and efficacy. We therefore observed ATP levels in combinations of NRTIs with 150μM RTV. All the NRTIs with 150μM RTV caused a decrease in ATP levels compared to the control. Additionally, RTV + d4T or FLT decreased ATP levels compared to the NRTI alone.
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2.4 Compiling mitochondrial toxicity parameters

To simplify the read-out of each mitochondrial toxicity experiment (mtDNA copy number, mitochondrial morphology, and ATP levels) we generated heat maps to visualize the ‘toxicity potential’ of each NRTI and PI combination (see Materials and Methods). MtDNA copy number was chiefly affected by combinations involving either FLT or SQV. Mitochondrial area was particularly affected by combinations involving ddC, mitochondrial solidity by combinations of IDV with either AZT or ddl, and mitochondrial entropy was predominantly affected by combinations with AZT, d4T or ddl. ATP levels were most severely affected by combinations involving IDV, NFV, or RTV (Figure 5). Compiling each individual mitochondrial morphology descriptors into one heat map showed that combinations involving FLT or ddl had more potential to be toxic than AZT, d4T, or ddC. In particular ddl + RTV and FLT + NFV showed high toxicity potential. AZT + RTV and d4T + NFV appeared to be the least toxic combinations (Figure 6).

Figure 5. Toxicity potential profiling of individual mitochondrial parameters. Toxicity potential was calculated as described in the Material and Methods.
3. Discussion

To our knowledge this is the first systematic in vivo screening of first-generation NRTI and PI combinations. By analysis of mtDNA copy number, mitochondrial morphology descriptors, and ATP levels, we have shown that combinations of NRTIs and PIs vary greatly in their ability to significantly alter any one of these selected mitochondrial toxicity parameters.

3.1 mtDNA copy number

In this study we observed that mtDNA copy number was particularly affected after 72h exposure to combinations of PIs with FLT or ddI, or SQV in combination with any NRTI (Figure 1 & 5). The detrimental effect of combinations including FLT was not surprising as FLT has consistently been found to severely deplete mtDNA copy number (Figure 1; Chapter 2 & 3). ddI is one of the first-generation NRTIs known to have high affinity for polymerase-\(\gamma\)\(^{21}\). We observed that ddI alone had marginal effects on mtDNA copy number, yet in combination caused marked changes (Figure 1). Combining ddI with IDV has previously been observed to be toxic and patients receiving this combination require close monitoring and frequent dose adjustments to avoid side effects\(^{442}\). Contraindication of combinations of IDV with other NRTIs has, to our knowledge, not been reported in the literature. Combining SQV with NRTIs also showed high potential for altering mtDNA levels (Figure 5). This is surprising as combinations of SQV with either AZT or ddI have been recommended for therapy naive adult patients\(^{139}\), and SQV is considered to be associated with mild drug interactions compared to other first-generation PIs\(^{400}\).

The polymerase-\(\gamma\) theory describes that the inhibition on polymerase-\(\gamma\) by NRTIs can lead to a decrease in mtDNA copy number\(^ {47}\). We, however, previously demonstrated that in C. elegans a direct correlation between exposure to some NRTIs and a decrease in mtDNA copy number does not exist (Chapter 3 & 4)\(^ {51}\). Additionally, in chapter 5, we showed that exposure to PIs can affect mtDNA copy number and that this effect is likely not dependent on direct polymerase-\(\gamma\) inhibition. It is possible that the observed depletion of mtDNA copy number upon exposure to SQV + NRTIs in this study is a nematode specific response. Nonetheless, it would be interesting to unravel why SQV in combination with NRTIs has such a strong additive effect on mtDNA copy number, especially so as combinations with SQV appear to have little effect on mitochondrial morphology or ATP levels compared to the NRTI alone.
3.2 Mitochondrial morphology

Variations in cellular bioenergetics have been shown to rapidly change mitochondrial morphology, for instance a reduction in $\Delta \Psi_{mt}$ can cause swollen mitochondria and a fragmented mitochondrial network. Mitochondrial morphology assessment after 72h exposure showed that combinations of PIs with ddC increased mitochondrial area compared to the control (Figure 2 A, D, G, & J, & Figure 5), indicating a significant decrease in mitochondrial energetics. ddC has been found to directly inhibit MRC complex I function, and our results suggest that this effect may be exacerbated by concurrent PI exposure.

Mitochondrial morphology has been shown to be required for mtDNA stability, and can be quantified by the solidity metric. We previously demonstrated that mitochondrial morphology becomes more fragmented by 200μM AZT and that mitochondrial network increases upon exposure to 200μM FLT or d4T after 72h exposure in C. elegans. This is similar to the results shown in this study, although the changes only reached statistical significance with 200μM. Combining IDV with either AZT or ddI increased mitochondrial solidity, signifying a decrease in mitochondrial fusion (Figure 2B). IDV alone, however, also increased mitochondrial solidity, indicating that the effect of IDV in combination with AZT or ddI is dependent on IDV and not the NRTI. d4T, ddC, and FLT negated the increase in mitochondrial solidity caused by IDV, which may point towards antagonistic effects of these antiretrovirals.

Entropy is a statistical measure of randomness and in the body-wall muscle of the C. elegans strain glo-1(zu391), mitochondria are neatly organised between the muscle cells and give a low measure of entropy. Combinations involving AZT, d4T, and ddI caused the most changes in mitochondrial entropy. Combining PIs with d4T or AZT generally normalised the higher order of entropy induced by either the PI or the NRTI alone, suggesting an antagonistic interaction. Combinations with ddI generally exacerbated the entropy of mitochondrial morphology (Figure 2 C, F, I, & L, & Figure 5).

3.3 ATP levels

ATP levels are a good measure of mitochondrial fitness. In this study we quantified ATP levels directly after exposure to drug combinations so as to assess their immediate effect on the mitochondria. The exact mechanism underlying the ability of PIs to rapidly inhibit mitochondrial function and consequently reduce ATP levels has yet to be discovered. Reyskens and Essop proposed that PIs induce a rapid rise in ROS production, and in this way can diminish $\Delta \Psi_{mt}$ and inhibit mitochondrial function (Chapter 5). NRTIs, on the other hand, have been proposed to directly inhibit complexes of the mitochondrial respiratory chain and thus reduce ATP levels (Chapter 3).

ATP levels were generally lower for combinations of RTV with any NRTI than either the control or the NRTI alone (Figure 3C) and supports the clinical observation that RTV is very toxic in high concentrations. Combinations with SQV showed no changes in ATP levels (Figure 3D) which is in accordance with SQV’s association with only mild drug interactions. Especially remarkable were the observations that combinations with IDV showed increased ATP levels compared to IDV or the NRTIs alone. In the case of IDV with either d4T or ddI, ATP levels were even higher than the control (Figure 3A). One study demonstrated that IDV + AZT caused a
reduction in ATP levels, which is in contradiction to our results. In this study, however, human umbilical vein endothelial cells were exposed to IDV + AZT for 72h as opposed to 1:50 minutes which explains these differences. The observed rise in ATP levels above those of the control may indicate that there is a decrease in ATP-consuming processes as the rate of energy production in the cell is always equal to the rate of energy expenditure. It is still unclear how rapid changes in mitochondrial function can influence long-term events; however, accelerated ageing has been related to a decrease in oxidative phosphorylation which suggests that the rapid decrease in ATP levels caused by antiretrovirals may underlie the occurrence of premature and accelerated ageing in HIV-1 patients (Chapter 3).

3.4 Boosting HAART

Drug interactions can alter the absorption, distribution, metabolism, or excretion of the administered compounds. Moreover, the pharmacologic actions of each drug can be altered through additive, synergistic, or antagonistic effects, which collectively can result in altered drug bioavailability and consequently toxicity and the occurrence of adverse side effects. RTV is known to be extremely toxic in high concentrations and is therefore rarely used for its potential to inhibit the viral protease. Instead, low concentrations of RTV are used to ‘boost’ HAART as RTV is known to increase the bioavailability of concomitant drugs. Combinations of NRTIs with 150μM of RTV showed less drastic ATP level decline than with 300μM RTV, except in the case of FLT where ATP levels lowered further (Figure 4). As we quantified ATP levels 1:50 min after drug exposure, we suspect that the effects of drug metabolism and excretion will be minimal. Nonetheless, it will be interesting to evaluate the extent to which NRTI and PIs can cause mitochondrial toxicity when their concentrations are changed as this may give us valuable insight into the pharmacologic actions of each combination.

3.5 Compiling mitochondrial toxicity parameters

By compiling the results from each established technique to assess mitochondrial toxicity we found that in C. elegans, FLT + NFV, ddi + RTV, and d4T + SQV were the combinations with the highest potential to generate mitochondrial toxicity. Combinations of AZT + NFV, RTV, or SQV, combinations of d4T + IDV or NFV, and combinations of ddC + NFV or SQV, had the lowest potential to generate toxicity (Figure 6). In support of these observations, IDV & NFV are considered to be generally well tolerated compared to RTV and SQV.

Translation of the compiled mitochondrial toxicity parameters, however, needs to be done with caution even though it provides great visual aid. The profiling of each mitochondrial toxicity parameter is based on statistical differences between the controls and the NRTI ‘backbone’, and does not take into account the possible benefit of the combination above singular drug exposure. For example, combinations of IDV with NRTIs generally normalise or even increase ATP levels compared to either IDV or the NRTIs alone (Figure 3), and the physiological response to this may be beneficial as an appose to detrimental. Additionally, the exposure time and drug concentrations differ greatly between experiments. For instance, mtDNA copy number and mitochondrial morphology were assessed after 72h, whereas ATP levels were quantified after just 1:50 minutes. Finally, each mitochondrial toxicity parameter, although related, portrays a different facet of the physiological response to drug exposure and should therefore be weighed according to its capacity to reflect adverse events. We have chosen a more crude method, however, to simply illustrate the potential of...
combining these techniques. Making use of a single parameter, like mtDNA copy number \(^{146}\), is insufficient to relay the true toxicity potential of the administered drug (Chapter 2 & 3). Our compilation approach therefore emphasizes the need to monitor multiple toxicity parameters in the clinic, so that clinicians are better equipped to predict the occurrence of adverse events and provide the patient with a suitable therapy regimen.

### 3.6 Research prospective

Future research into combinations of second-generation antiretrovirals and common combinations of two first-generation NRTIs or PIs will hasten current advances in regimen discovery. Nowadays, first generation NRTIs and PIs are seldom recommended in the initial treatment of HIV-1 infection due to the high incidence of adverse events affiliated with their use \(^{347,444}\). However, first generation antiretrovirals are gaining interest as ‘salvage therapy’ to combat drug resistance \(^{445}\). For example, the combinations of SQV with either NFV or RTV to bolster current therapy regimens have received renewed attention as they have modest pharmacological interactions and are well tolerated. On the contrary, IDV with either NFV or SQV has been shown to have little benefit above other PI combinations, including their potential to induce adverse events \(^{400}\). Combinations of NRTIs are frequently used as a PI sparing regimen, yet some combinations have proven more toxic than others. Combining ddC with d4T in vitro, for instance, has shown enhanced lactate production and mtDNA decline \(^{446}\).

Moreover, combining ddI with d4T in the clinic has been found to increase the risk of lactic academia, pancreatitis, and peripheral neuropathy \(^{24}\), yet interestingly this combination is still recommended for the initial treatment of therapy naive patients \(^{441}\).

More research is warranted to discover both dangerous or well tolerated combinations, particularly in the face of increased drug resistance where the only retort is often treatment with ‘multidrug’ regimes that can contain up to five drugs \(^{447}\). Unfortunately, the use of certain antiretroviral combinations is also dependent on the regimen cost, especially in low-income countries where cheaper generic and often first-generation drugs are continuously sought after \(^{448}\). *C. elegans* poses an attractive system for high-throughput screening of multiple complex regimens and can assist in diminishing the prescription of drug combinations that unfavourably interact with each other and have increased potential to induce adverse side effects.
4. Materials and Methods

4.1 Strains and Conditions

*C. elegans* strains used were N2 Bristol wild-type strain, and glo-1(zu391) using mito::GFP expressed from the myo-3 promoter. Using alkaline hypochlorite treatment of gravid adults, synchronous populations of strains were obtained. Eggs were allowed to hatch overnight at 20°C in M9 buffer and L1 larvae were placed on NGM plates which were pre-seeded with *E. coli* OP50 as a food source until L4. Unless otherwise mentioned, L4 animals were then transferred to NGM plates containing the compounds of interest.

4.2 Quantitative real time PCR

Quantitative real time PCR was performed as described by de Boer. In short, synchronized wild type (N2), 2h post L4 molt, young adult worms were transferred to OP50 seeded FUdR NGM plates containing antiretroviral combinations. Five adult worms were collected 72h drug exposure and lysed in Lysis buffer (50mM KCl, 10mM Tris (pH 8.3), 2.5mM MgCl2, 0.45% NP-40 (IGEPAL), 0.45% Tween-20, 0.01% Gelatin, 20mg/mL Proteinase K). Before detection in the PCR, the solution was diluted 40 times and 2 μl was used as input in the PCR reaction. Primers specific for cytochrome c oxidase subunit I (COX1) were used for the determination of mtDNA copy number. PCRs were performed using the Taqman® universal cycling conditions with amplified products being detected using a Taqman® probe for CeCOX1. Fluorescent signal intensities were determined using the 7300 Real-Time PCR System (Applied Biosystems) with software SDS (version 1.9.1). To quantify the absolute quantity of mtDNA per worm, a standard curve was generated from a plasmid with a fragment of the cox1 gene. After PCR the total mtDNA copies per worm were calculated with at least two biological and two technical replicates.

4.3 Image acquisition and analysis of mitochondrial morphology

Mitochondrial morphology in body wall muscle cells was visualized in transgenic glo-1(zu391) animals using mito::GFP expressed from the myo-3 promoter making it ideal for the analysis of mitochondrial morphology. Synchronized worms were allowed to develop until L4, after which they were transferred to plates with drugs added to the NGM. 72h later at least 10 individual worms were imaged using the Nikon A1 confocal microscope, with a Plan Apo 60x WI objective with a numerical aperture of 1.27, a 488nm argon laser, a pixel size of 0.2μM and NIS-Elements AR v4.1004 (Build 854) software. Image processing was performed in ImageJ freeware (W.S. Rasband, U.S.A. National Institutes of Health, Bethesda, Maryland, USA, http://rsb.info.nih.gov/ij/, 1997–2012). Mitochondria were segmented by means of a custom-designed image-processing pipeline (available at www.limid.ugent.be/downloads). First, uninformative slices or slices with reflections were removed from the image stacks, using an image quality criterion that only retains slices with a covariance >1. The triaged stacks were then projected according to the maximum pixel intensity and preprocessed by background subtraction (rolling ball radius 15) and local contrast enhancement (block size 15) after which objects were enhanced by means of a multi-scale Laplacian operator. Subsequently, the image was binarized according to a Yen autothresholding procedure and the resulting mask was used for analyzing shape and intensity metrics of objects larger than a predefined size (>12 pixels) on the original image.
addition, global texture metrics were measured on the original image using the GLCM texture plugin (by Julio Cabrera).

For unsupervised clustering, a subset of mitochondria- and image-specific descriptors was retained. Of the segmented mitochondria, average values were calculated for Mean (average grey value), Area (average projected area), AR (aspect ratio of the fitted ellipse, i.e. major axis/minor axis), Feret (longest internal distance), Solidity (area/convex area), Circularity (\((4\pi\text{area}/\text{perimeter}^2)\)) and Roundness (\((4\pi\text{area}/\text{major axis}^2)\)). Of the texture parameters, the following metrics were calculated as averages from a horizontal and a vertical gray-level co-occurrence matrix (GLCM), both with one pixel offset.: Entropy\(\sum_{i,j=0}^{N-1} P_{ij} \left(-\ln P_{ij}\right)\), Contrast \(\sum_{i,j=0}^{N-1} P_{ij} (i-j)^2\) and Correlation \(\sum_{i,j=0}^{N-1} P_{ij} \frac{(i-\mu_i)(i-\mu_j)}{\sigma_i \sigma_j}\), with \(P_{ij}\) reflecting values of row \(i\) and column \(j\) in the GLCM.

Statistical analyses were performed in Microsoft excel 2010\textsuperscript{tm}, Matlab 2010a (Mathworks, Eindhoven, The Netherlands) or R freeware. Individual parameters (shape and texture descriptors) were statistically compared between conditions, by means of pairwise student’s T-tests or, in case of non-normal distributions, Wilcoxon rank sum tests. Results were summarized in boxplots.

### 4.4 ATP measurements

ATP luminescence measurements were adapted from Lagido et al. (2008). All measurements were performed in CPB (pH 6.5) with a final volume of 100μL. 10μL of a 10x concentrated drug solution was pipetted into white flat-bottomed 96-wells plates (Greiner). Each condition was measured in at least 3 biological replicates and at least 8 technical replicates. Luminescence was measured in a Biotek Synergy MX plate reader in the visible spectral range (300-600nm). Young adult worms grown at 25°C were washed off FUdR plates with S-basal and collected in CPB. Luminescence buffer was prepared with 1mM D-Luciferin and 0.05% Triton-X, to improve cuticle permeability (all final concentrations, in CPB). Luminescence was measured continuously during 2 minutes. The average of green fluorescent protein (GFP) expression was measured continuously with a 485/520nm filter set for 1 minute (12 times) right after the luminescence measurement for normalisation. The different biological replicates were analysed with a two-way ANOVA with replication with a significance value of \(p \leq 0.05\). The mean ± standard error is reported.

### 4.5 Compiling toxicity parameters

Toxicity potential for individual mitochondrial toxicity parameters was calculated by annotating values, independent of directional change, to statistically generated asterisks as follows: Drug combinations with a P-value of <0.05; <0.01; <0.001 compared to the control (\(\times\)) were annotated with a value of 1, 2, or 3 respectively. Drug combinations with a P-value of <0.05; <0.01; <0.001 compared to the NRTI alone (\(\times\)) were annotated with a value of 1, 2, or 3 multiplied by 2, respectively. Values derived from drug combinations that were statistically different to both the control (\(\times\)) and the NRTI alone (\(\times\)\(\times\)) were added together:
Toxicity potential for all the mitochondrial toxicity parameters together was calculated by assuming equal weight for mtDNA copy number, mitochondrial morphology, and ATP levels. As mitochondrial morphology could be split into 3 separate parameters (area, solidity, and entropy) the sum of all individual parameters was divided by 3. RTV ‘booster’ results were not included in the compilation.