Towards an understanding of the side effects of anti-HIV drugs using Caenorhabditis elegans
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Chapter 5

Protease Inhibitor HIV-1 antiretroviral therapy causes immediate mitochondrial respiratory chain dysfunction that can be attenuated by antioxidants
Protease Inhibitor HIV-1 antiretroviral therapy causes immediate mitochondrial respiratory chain dysfunction that can be attenuated by antioxidants

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Abstract

The introduction of HIV-1 protease inhibitors (PI) was considered a major advance in anti-retroviral therapy as their implementation has considerably lowered the mortality rate of HIV-1 patients and strengthened modern therapy regimens. PIs, however, are known to cause severe adverse side effects which are proposed to originate from mitochondrial toxicity. To date, the precise cause and chronology of PI induced adverse events, and the role of mitochondria in this process remains unknown. In this study we combine Caenorhabditis elegans as an established model system with molecular-biological and functional assays, to further elucidate PI induced toxicity. We conclude that PIs cause rapid mitochondrial respiratory chain dysfunction which is reversible upon supplementation with antioxidants, pointing towards ROS as the active agents in this process. Chronic exposure to PIs causes changes in mitochondrial network morphology, fitness, and fecundity. Taken together, these results support an important role for mitochondria in PI induced adverse events.

1. Introduction

1.1 HIV-1 protease inhibitors

HIV-1 Protease inhibitors (Pis) are a frequently prescribed drug class in antiretroviral therapy and are routinely administered together with the Nucleoside Reverse Transcription Inhibitor (NRTI) class in Highly Active Anti-Retroviral Therapy (HAART). PIs competitively bind the viral aspartyl-protease active site with high affinity and therefore inhibit cleavage of viral polypeptides and subsequent maturation of HIV-1. In this way PIs hinder viral replication and consequent infection of other cells. Because of their clinical potency, PIs led to increased viral suppression and a significantly lower mortality rate of HIV-1 patients. Their introduction was therefore considered a major advance in anti-retroviral therapy. It quickly became apparent, however, that PIs could cause (severe) adverse side effects.
1.2 PIs induced adverse events and mitochondrial toxicity

In general, PIs have been linked to various side-effects ranging from mild – such as gastrointestinal irritations, headache and fatigue – to severe, such as cardiovascular complications and metabolic abnormalities including hyperlipidemia, lipodystrophy, and insulin resistance. The more severe symptoms are usually observed after chronic PI based therapy. PI adverse events, much like adverse events from NRTIs, are similar to those observed in patients suffering from mitochondrial diseases, implying that PIs induce mitochondrial dysfunction. In support of this, recent studies have demonstrated that PIs affect mitochondrial membrane potential (ΔΨm), mitochondrial morphology and expression levels of mitochondrial DNA (mtDNA) encoded mitochondrial respiratory chain (MRC) subunits. PIs have also been shown to induce elevated mitochondrial reactive oxygen species (ROS) production, possibly via depolarization of mitochondrial membrane potential or by decreasing Cu/ZnSOD gene expression. The precise cause for PI induced mitochondrial toxicity, however, is - to date - unclear.

1.3 ROS as a central theory in PI induced mitochondrial toxicity

A principal theme within PI-induced mitochondrial dysfunction is the elevation of ROS production. Several human and animal studies have clearly established a link between PI exposure and increased ROS production in different cell- and tissue types (reviewed in Reyskens & Essop, 2014). For example, the widely used PI and antiretroviral ‘booster’ ritonavir has been shown to cause increased oxidative stress in arterial endothelium, and through this mechanism is expected to stimulate cardiovascular disease. Although most studies suggest a mitochondrial origin for PI induced ROS production, others have indicated the presence of extra-mitochondrial ROS and hypothesize that cytosolic ROS is the initial trigger that leads to mitochondrial malfunction. For instance, ritonavir treated porcine carotid arteries showed elevated levels of ROS likely through increased NAD(P)H oxidase activity which was followed by an elevation in superoxide production. The exact role of oxidative stress and the chronology of events that lead to mitochondrial dysfunction, however, are still unknown.

1.4 Aim of this study

In comparison to NRTIs, little research has been done to categorize and elucidate PI induced toxicity. In this study, we focus on the ability of PIs to induce elevated ROS production as a major mechanism underlying their toxicity. We use Caenorhabditis elegans together with verified molecular-biological techniques to specifically study the mechanisms and timing of PI induced mitochondrial toxicity. C. elegans has several advantages over other model systems as it is highly malleable and has frequently been used for the elucidation of molecular pathways implicated in many human diseases, including those of the mitochondria. Not only is C. elegans a very practical system, this nematode has also been used before to study antiretroviral drug-specific impact on mitochondria.
HIV-1 protease inhibitors cause immediate mitochondrial dysfunction

2. Results

To investigate the mechanisms behind PI induced toxicity, we selectively chose first-generation PIs – namely saquinavir (SQV), ritonavir (RTV), indinavir (IDV) and nelfinavir (NFV) – (Table 1) as these drugs have been studied most, have been shown to regularly cause adverse events, and are still prescribed in the clinic today.

Table 1. First generation PIs used in this study. The approval date indicates FDA approval date.

<table>
<thead>
<tr>
<th>Antiretroviral drug class</th>
<th>Drug name</th>
<th>Other names/Abbreviations</th>
<th>Approval date</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protease Inhibitor (PI)</td>
<td>Indinavir</td>
<td>IDV</td>
<td>1996</td>
</tr>
<tr>
<td></td>
<td>Nelfinavir</td>
<td>NFV</td>
<td>1997</td>
</tr>
<tr>
<td></td>
<td>Ritonavir</td>
<td>RTV</td>
<td>1996</td>
</tr>
<tr>
<td></td>
<td>Saquinavir</td>
<td>SQV</td>
<td>1995</td>
</tr>
</tbody>
</table>

2.1 Selected PIs do not affect E. coli growth at relevant concentrations

We cultured *C. elegans* on lawns of the frequently used nematode food source *Escherichia coli* OP50 which were seeded on nematode-growth-media agar plates containing the PIs of interest. A search of the literature revealed that the bactericidal or bacteriostatic properties of HIV-1 PIs on *E. coli* have not been tested. We therefore set out to test if PIs inhibit growth of *E. coli* OP50 and the commonly used Gram-positive bacterium *Bacillus subtilis 168*. No growth perturbations were found on *E. coli* OP50 or *B. subtilis 168* at relevant PI concentrations (≥300μM) (data not shown).

2.2 ROS detoxification enzyme expression is altered upon exposure to PIs

PI induced ROS production has been suggested to be caused by a rapid decrease in expression of enzymes that regulate redox homeostasis. We therefore measured *gst-4* and *sod-3* expression during short-term PI exposure. Glutathione S-transferases (GSTs) are involved in detoxification of superoxide radicals, endogenous toxic metabolites and exogenous toxic chemicals. *GST-4* is nematode specific and has been shown to be directly involved in resistance to oxidative stress. During six hours of continuous exposure to IDV and NFV, *gst-4* expression in *C. elegans* showed a trend towards an initial decline followed by an increase, and RTV and SQV showed a trend towards decreased *gst-4* expression (Figure 1A). The decreased expression of *gst-4* worsens for RTV and SQV during the first 3h exposure, gradually returning to levels comparable to the control after 6h. The acute decrease in *gst-4* expression induced by IDV and NFV rapidly normalized within 2h and continued to rise above that of the control during the 6h exposure (Figure 1A). PIs were dissolved in dimethyl sulfoxide (DMSO). 3% DMSO showed a small yet significant change in *gst-4* expression at 5h (Figure 1B).
Figure 1. Relative \( \text{gst-4} \) expression during PI exposure. Relevant DMSO concentrations in d\( \text{H}_2\text{O} \); A: IDV & NFV = 0.3%; B: RTV & SQV = 3%. PIs in Citrate Phosphate Buffer (CPB); C: IDV; D: NFV; E: RTV; F: SQV. Results depict 6 hours continuous exposure of therapy naïve animals to 300\( \mu \text{M} \) PIs. Significance was calculated using a multifactorial ANOVA without replication, * = \( P<0.05 \).

SOD-3 is a manganese dependant mitochondrial-matrix specific enzyme that catalyses the dismutation of superoxide to oxygen and hydrogen peroxide \(^{262}\). Additionally, SOD-3 has been proposed to be closely associated with the MRC I:III:IV supercomplexes; supporting their stability and detoxifying oxygen radicals produced at these sites \(^{265,261}\). Firstly, we measured \( \text{sod-3} \) expression after short-term exposure (4h) to IDV. Short-term exposure showed no change in \( \text{sod-3} \) expression (Figure 2A). Second, we measured \( \text{sod-3} \) expression after chronic exposure (72h) to IDV. IDV caused a concentration dependent trend towards an increase in \( \text{sod-3} \) expression (Figure 2B). Taken together, these results suggest that short-term PI exposure influences cytosolic but not mitochondrial ROS detoxification enzyme expression. Additionally, only after prolonged exposure to PIs is there an increased production of ROS in the mitochondria.

Figure 2. \( \text{sod-3::GFP} \) fluorescence intensity change in response to IDV exposure. Only after chronic exposure of animals to IDV did mitochondrial ROS production increase. A: 4h exposure, B: 72h exposure. IDV exposure is not significantly different compared to control for both time points and concentrations. The y-axis denotes GFP expression (arbitrary units).
2.3 Reactive oxygen species production rate is decreased by PI exposure

PIs appear to cause a rapid decrease in gst-4 expression which may cause a rise in ROS. We therefore measured ROS levels during short-term exposure to PIs. The production rate of general ROS, measured with H$_2$DCFDA during 4h PI exposure, declined significantly (Figure 3). Upon further analysis of general ROS production we found a trend that the production of ROS upon exposure to PIs declined within approximately 10 minutes (Figure 4).

![Figure 3](image-url)

**Figure 3.** Relative average global ROS production rate over 4 hours in response to PI exposure. Global ROS levels were measured by quantifying the fluorescence reporter dye H$_2$DCFDA in vivo during 4 hours exposure of naïve animals to PIs. Statistics were calculated by a two sided student’s t-test assuming unequal variance, compared to control. * = P<0.05, ** = P<0.01, *** = P<0.001. Error bars indicate standard error.

![Figure 4](image-url)

**Figure 4.** General ROS production of therapy naïve animals during 30min drug exposure, relative to control. IDV [A], NFV [B], RTV [C], and SQV [D] showed a trend towards a decrease in ROS production. ROS levels were measured by quantifying the fluorescence reporter dye H$_2$DCFDA in vivo. Error bars indicate standard error. PI exposure is not significantly different compared to control at all the time points.

2.4 ATP production and oxygen consumption rate are rapidly perturbed upon exposure to PIs

A reduction of general ROS, measured by H$_2$DCFDA, can be regarded as a reduction in total ROS flux, coinciding with a decreased metabolism and a lower hydrogen peroxide production rate. In support of this, lower ATP levels were previously observed after exposure to IDV, NFV, and SQV. To assess if MRC function is inhibited, we measured ATP levels in vivo upon exposure to increasing concentrations (200μM, 300μM, and 500μM) of PIs. After 2.5 minutes exposure to therapy naïve animals using 200μM, RTV was the only PI that caused lower levels of ATP, whereas with 300μM, all PIs showed significantly decreased ATP levels. With 300μM RTV, however, a dose dependant decrease in ATP levels was not evident. 300μM RTV caused higher ATP levels than that caused by 200μM. Upon increasing PI exposure to 500μM, a continued concentration dependent decline
was only observed for SQV. ATP levels with 500μM IDV and NFV returned to levels comparable to the control. 500μM RTV showed lower ATP levels than with 300μM, yet not lower that that caused by 200μM (Figure 5A).

To further assess MRC dysfunction, we measured oxygen consumption rates upon exposure to 300μM IDV. Within 5 minutes, oxygen consumption rate was significantly reduced (Figure 5B). Taken together, these results suggest that PIs cause rapid MRC dysfunction.

2.5 PIs affect mitochondrial morphology

Various studies have demonstrated that PIs affect mitochondrial morphology \(^{96,97,106}\). A decrease in ATP production and an increase in ROS can lead to changes in mitochondrial morphology, specifically mitochondrial network fragmentation \(^{413}\). In light of this and our previous results we surmised that PIs may affect mitochondrial morphology. Quantitative mitochondrial morphology analysis after 72h exposure revealed that PIs caused significant disruption of mitochondrial networks (Figure 6).

Compared to control animals, IDV caused the most distinct changes in mitochondrial morphology, visible as significantly decreased mitochondrial area and increased circularity and solidity; all indications that mitochondrial networks are more fragmented. Additionally, IDV caused significant changes in mitochondrial organisation, as measured by the entropy metric (Figure 6B, G-H, & J). NFV also caused significant changes in circularity and solidity, indicating mitochondrial network fragmentation (Figure 6C, & F-I). RTV showed only a significant increase in entropy (Figure 6D, & I-J), and SQV only a decreased mitochondrial area (Figure 6E, F, & J) compared to control.
HIV-1 protease inhibitors cause immediate mitochondrial dysfunction

Figure 6. Chronic exposure to PIs caused changes in mitochondrial morphology. A-E: Mitochondrial morphology in the body wall muscle of worms exposed to PIs (300 μM) for 72h (A: Control, B: IDV, C: NFV, D: RTV, E: SQV). F-I: Boxplots comparing selected metrics per condition. Asterisks indicate statistically different changes compared to the control (per metric). J: Two-dimensional clustergram performed on the standardized dataset (z-scores) of features extracted after image analysis, using Euclidean distance as distance metric and the average value as linkage value for the dendrograms.

2.6 Short term PI exposure leads to alternate levels of mtDNA

Changes in mtDNA quality and copy number have been shown to influence MRC function, mitochondrial ROS production and mitochondrial morphology. Although PIs have not been shown to change mtDNA quality or copy number, we nonetheless wanted to rule out the possibility that PI induced mtDNA replication inhibition was the cause for the observed changes in mitochondrial morphology and MRC function. To assess mtDNA replication defects, we performed two-dimensional neutral/neutral agarose gel-electrophoresis (2DNAGE) in Human Embryonic Kidney 293 (HEK293T) cells after 48h exposure to IDV, and to quantify mtDNA copy numbers after short-term and chronic exposure we performed a mtDNA qPCR in C. elegans using the conserved mtDNA encoded COX1 gene as a target (see Materials & Methods).
IDV did not induce mtDNA replication stalling (Figure 7) and PIs did not distinctly reduce mtDNA copy numbers after chronic exposure (Table 3). Only after 48h and 72h did IDV and SQV respectively cause significantly increased and decreased mtDNA copy number. Short-term exposure to PIs, however, caused significant changes in mtDNA copy number compared to the control (Table 4). After 1h all the PIs induced a significant rise in mtDNA copy number. This rise was rapidly abrogated to values lower than the control at 2h and 3h; significantly so for RTV and SQV. At 4h, mtDNA copy number returned to levels comparable to the control, yet after 5 hours a rise was again apparent, which was significant for IDV and NFV. At 6h mtDNA copy numbers normalised for all PIs. Taken together, we can conclude that the changes in mitochondrial morphology upon chronic exposure to PIs are likely not caused by mtDNA replication defects or by a change in mtDNA copy number. Short term exposure to PIs caused mitochondrial mtDNA fluctuations that were rapidly normalised; indicating that PI induced changes in MRC function may affect mtDNA replication.

Table 4. mtDNA copy numbers rose rapidly and eventually normalized during short-term PI exposure. Relative quantities (%) of mtDNA compared to control animals. * = P-value <0.05, ** = P-value <0.01, *** = P-value <0.001. Significance was determined using a two-tailed student’s t-test assuming unequal variance compared to control animals. Numbers between parentheses indicate 95% confidence intervals (51df).

<table>
<thead>
<tr>
<th></th>
<th>1h</th>
<th>2h</th>
<th>3h</th>
<th>4h</th>
<th>5h</th>
<th>6h</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(+/−)</td>
<td>(+/−)</td>
<td>(+/−)</td>
<td>(+/−)</td>
<td>(+/−)</td>
<td>(+/−)</td>
</tr>
<tr>
<td>Control</td>
<td>100 (19/27)</td>
<td>100 (21/27)</td>
<td>100 (21/27)</td>
<td>100 (21/27)</td>
<td>100 (21/27)</td>
<td>100 (21/27)</td>
</tr>
<tr>
<td>IDV</td>
<td>175 (33/41) ***</td>
<td>100 (21/27)</td>
<td>74 (15/19) *</td>
<td>110 (24/30)</td>
<td>157 (33/43) **</td>
<td>98 (20/25)</td>
</tr>
<tr>
<td>NFV</td>
<td>241 (32/65) ***</td>
<td>84 (18/23)</td>
<td>80 (17/22)</td>
<td>103 (19/23)</td>
<td>150 (32/41) **</td>
<td>111 (24/30)</td>
</tr>
<tr>
<td>RTV</td>
<td>137 (28/34) ***</td>
<td>74 (15/19) *</td>
<td>66 (14/18) **</td>
<td>97 (21/26)</td>
<td>122 (24/31)</td>
<td>119 (25/32)</td>
</tr>
<tr>
<td>SQV</td>
<td>135 (29/37) ***</td>
<td>76 (13/16) *</td>
<td>73 (15/18) *</td>
<td>91 (19/25)</td>
<td>108 (23/29)</td>
<td>94 (20/26)</td>
</tr>
</tbody>
</table>
2.7 PIs affect fitness and fecundity of *C. elegans*

PIs have frequently been associated with the initiation or exacerbation of degenerative processes, diseases and syndromes. Many of these detrimental processes have been linked to mitochondrial dysfunction and specifically to oxidative stress. To assess if chronic PI exposure also affects fitness, we quantified thrashing rates and brood size in *C. elegans*. PIs significantly decreased thrashing rates at 24h, 48h, and 72h; with RTV and SQV having the most prominent effect (Table 5). Nematodes exposed to RTV and SQV also showed more spastic, uncoordinated movements than other conditions (data not shown). It is therefore important to note that, as we only quantified thrashing rates in animals showing constant rhythmic sigmoidal body bends, these results do not entirely represent the severity of RTV and SQV induced fitness decrease.

Table 5. PIs reduced the number of sigmoidal body bends, per worm, per minute. PI concentration = 300μM. Statistics were calculated by a two sided student's t-test assuming unequal variance, compared to the control of that same time point. Control 48h vs Control 24h, Control 72h vs Control 48h. ** = P<0.01, *** = P<0.001.

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>IDV</th>
<th>NFV</th>
<th>RTV</th>
<th>SQV</th>
</tr>
</thead>
<tbody>
<tr>
<td>24h</td>
<td>134.7 (+/−13.8)</td>
<td>125.8 (+/−21.2) **</td>
<td>122.6 (+/−25.7) ***</td>
<td>106.2 (+/−30.7) ***</td>
<td>99.6 (+/−27.9) ***</td>
</tr>
<tr>
<td>48h</td>
<td>125.9 (+/−15) ***</td>
<td>106.7 (+/−22.7) ***</td>
<td>109.1 (+/−19.1) ***</td>
<td>67.3 (+/−20.5) ***</td>
<td>82.1 (+/−26.2) ***</td>
</tr>
<tr>
<td>72h</td>
<td>111.6 (+/−14.4) ***</td>
<td>95.0 (+/−26.5) **</td>
<td>89.8 (+/−33.1) **</td>
<td>66.5 (+/−22.3) ***</td>
<td>50.2 (+/−31.9) ***</td>
</tr>
</tbody>
</table>

Mitochondrial function is essential for oocyte production and fecundity, and brood size is often used in *C. elegans* as a measure of fitness. We therefore quantified the number of progeny during exposure to PIs. NFV, RTV, and SQV caused a significant decline in brood size, whereas IDV showed no change (Figure 8).

![Figure 8. Total number of progeny during PI exposure. PI concentration = 200μM. Statistics were calculated by a two sided student’s t-test assuming unequal variance, compared to control. ** = P<0.01, *** = P<0.001.](image)

PIs use has also been associated with mitochondrial dysfunction induced premature and accelerated ageing. To see if PIs cause accelerated ageing we performed lifespan analyses of *C. elegans* exposed to PIs. Of the PIs tested, only SQV showed significant lifespan-shortening effects compared to the relevant DMSO control of 3% (Table 6). RTV and IDV had no effect. 3% DMSO, which is also the relevant control for RTV, caused a decrease in average and maximal lifespan. 0.3% DMSO, which is the relevant control for IDV, showed no effect on average lifespan, but did increase maximum lifespan.
Table 6. PIs and relevant DMSO concentrations altered average lifespan. PI concentration = 300μM. All animals are N2 at 20°C, exposed from L4. Mean and maximum refer to the amount of days after exposure. Statistical analysis of the PIs were conducted compared to their respective DMSO control (IDV = 0.3%; RTV & SQV = 3%). DMSO statistical analyses were conducted compared to the control. SEM = standard error of the mean.

<table>
<thead>
<tr>
<th>Exposure</th>
<th>N (total)</th>
<th>Mean ± SEM</th>
<th>Maximum ± SEM</th>
<th>P-value (Mantel-Cox test)</th>
<th>P-value (Gehan-Breslow-Wilcoxon test)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>442</td>
<td>18.0 (±0.5)</td>
<td>27.5 (±1.5)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.3% DMSO</td>
<td>304</td>
<td>18.0 (±2.0)</td>
<td>30.5 (±1.5)</td>
<td>0.0021</td>
<td>0.5074</td>
</tr>
<tr>
<td>IDV</td>
<td>153</td>
<td>17.0 (±0.0)</td>
<td>28.0 (±0.0)</td>
<td>0.0617</td>
<td>0.8564</td>
</tr>
<tr>
<td>RTV</td>
<td>166</td>
<td>17.0 (±0.0)</td>
<td>26.0 (±0.0)</td>
<td>0.0501</td>
<td>0.0787</td>
</tr>
<tr>
<td>SQV</td>
<td>92</td>
<td>14.0 (±0.0)</td>
<td>24.0 (±0.0)</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>RTV + NAC</td>
<td>275</td>
<td>16.5 (±1.5)</td>
<td>26.0 (±1.0)</td>
<td>0.9329</td>
<td>0.9942</td>
</tr>
</tbody>
</table>

2.8 Antioxidants can attenuate short-term ATP level decline and mtDNA copy number decrease

In their model for PI induced mitochondrial dysfunction, Reyskens and Essop proposed that the presence of PI induced cytosolic ROS and the related altered expression of redox-regulating enzymes are likely the initial triggers that lead to mitochondrial malfunction. To assess if the PI induced mitochondrial dysfunction we observed is dependent on the generation of ROS, we exposed therapy naïve animals to PIs and supplemented them with selected anti-oxidants (AOXs); l-ascorbic acid (Vitamin C, LAA), N-acetylcysteine (NAC), Trolox (a water soluble analogue of α-tocopherol: vitamin E), and acetyl-l-carnitine (ALCAR) (Chapter 3, Supporting Information, Table 6). We anticipated that supplementation with AOXs should alleviate the PI induced decrease in ATP levels. Indeed, upon supplementation of IDV with increasing concentrations of AOXs, ATP levels normalized (Table 7). NAC was the most effective AOX in alleviating the IDV induced ATP level decline, whereas Trolox was least effective. A stepwise increase in the concentration of AOXs above 100μM up to 10mM had little to no additional beneficiary effect.

To see if the rapid fluctuations in mtDNA levels were also dependent on ROS levels, we supplemented RTV with 100μM NAC and quantified mtDNA copy number. NAC significantly attenuated the RTV induced mtDNA copy number increase after 1h exposure. The NAC induced attenuation, however, resulted in mtDNA copy numbers similar to control animals at 0h (Figure 9A). 100μM NAC alone also decreased mtDNA copy numbers compared to 1h controls (Chapter 3, Supporting Information, Figure 4), suggesting that redox imbalance can strongly and rapidly affect mtDNA copy numbers.
HIV-1 protease inhibitors cause immediate mitochondrial dysfunction

Table 7. 300μM IDV decreased ATP levels were attenuated upon exposure to anti-oxidants. ATP levels were measured 2.5 minutes after exposure in vivo. Statistics were calculated with a two-way ANOVA with replication; IDV compared to control and IDV + anti-oxidant compared to IDV. * = P<0.05, ** = P<0.01, *** = P<0.001.

<table>
<thead>
<tr>
<th>Exposure</th>
<th>Relative ATP level</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>100 (±1)</td>
<td></td>
</tr>
<tr>
<td>300μM IDV</td>
<td>67 (±4)</td>
<td>*** (3.38E-09)</td>
</tr>
<tr>
<td>300μM IDV + 100μM NAC</td>
<td>118 (±11)</td>
<td>*** (6.84E-05)</td>
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<td>300μM IDV + 200μM NAC</td>
<td>122 (±10)</td>
<td>*** (3.97E-06)</td>
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<td>101 (±9)</td>
<td>*** (3.98E-04)</td>
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<td>102 (±10)</td>
<td>*** (2.19E-03)</td>
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<td>300μM IDV + 1mM LAA</td>
<td>54 (±8)</td>
<td>*** (5.35E-04)</td>
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<td>300μM IDV + 10mM LAA</td>
<td>108 (±7)</td>
<td>** (2.62E-06)</td>
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<tr>
<td>300μM IDV + 100μM ALCAR</td>
<td>102 (±8)</td>
<td>*** (1.09E-06)</td>
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<td>300μM IDV + 200μM ALCAR</td>
<td>84 (±8)</td>
<td>*** (4.05E-06)</td>
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<tr>
<td>300μM IDV + 500μM ALCAR</td>
<td>84 (±8)</td>
<td>*** (6.65E-04)</td>
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<tr>
<td>300μM IDV + 1mM ALCAR</td>
<td>90 (±8)</td>
<td>*** (5.53E-05)</td>
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<tr>
<td>300μM IDV + 10mM ALCAR</td>
<td>94 (±7)</td>
<td>** (1.13E-04)</td>
</tr>
<tr>
<td>300μM IDV + 100μM Trolox</td>
<td>78 (±7)</td>
<td>** (2.27E-01)</td>
</tr>
<tr>
<td>300μM IDV + 200μM Trolox</td>
<td>88 (±6)</td>
<td>** (1.39E-03)</td>
</tr>
<tr>
<td>300μM IDV + 500μM Trolox</td>
<td>92 (±8)</td>
<td>** (3.47E-03)</td>
</tr>
<tr>
<td>300μM IDV + 1mM Trolox</td>
<td>96 (±10)</td>
<td>** (1.05E-03)</td>
</tr>
<tr>
<td>300μM IDV + 10mM Trolox</td>
<td>63 (±6)</td>
<td>(8.82E-01)</td>
</tr>
</tbody>
</table>

Figure 9. PIs induced mtDNA copy number increase was attenuated by anti-oxidants. A: 300μM RTV increased mtDNA copy number compared to control nematodes after 1h exposure (light grey vs 1h Control). 100μM NAC attenuated this increase (dark grey vs light grey). mtDNA copy number increases during nematode development and therefore rose (Control 0h vs Control 1h). B: The slight decrease in mtDNA copy numbers caused by 72h exposure to 300μM IDV was attenuated upon supplementation with 100μM AOXs. Error bars show the 95% C.I. (51df). Significance was determined using a two-tailed student’s t-test assuming unequal variances. * =P-value <0.05, ** = P-value <0.01, *** = P-value <0.001. A: control animals at 1h compared to RTV, and RTV + NAC compared to RTV, and Control 1h vs Control 0h; and B: IDV + AOX compared to IDV.

Chronic exposure to PIs caused fluctuations in mtDNA copy number for IDV and SQV (Table 3). To see if the changes in mtDNA copy number during chronic exposure were also dependent on ROS, we supplemented IDV with 100μM AOX and quantified mtDNA copy number after 72h exposure. The slight decline in mtDNA copy number caused by IDV showed a clear trend towards normalisation when animals were supplemented with NAC, LAA, and ALCAR. Trolox was able to significantly attenuate the decline in mtDNA copy number (Figure 9B).
2.9 Anti-oxidants may attenuate the PI induced decrease in fitness

As supplementation with AOXs could attenuate the short-term decrease in mtDNA copy numbers and ATP levels induced by PI exposure, we wondered if the decrease in fitness caused by long-term exposure to PIs could also be rescued. Supplementation of IDV with AOXs showed varying degrees of improving the decrease in thrashing rate caused by IDV. At 24h exposure, ALCAR and Trolox significantly attenuated thrashing rates, along with NAC at 48h. This rescue was, however, lost after 72h exposure. LAA showed a trend towards attenuation of thrashing rates, which was strongest at 72h (Table 8). Besides fitness we also tested the ability of AOXs to change lifespan during PI exposure. Lifespan was not significantly affected on exposure to RTV alone, and supplementation of RTV with NAC also had no effect (Table 6). Taken together, AOXs have no clear effect on long-term PI induced reduction in fitness.

Table 8. Anti-oxidants sometimes attenuated the decrease in fitness caused by IDV. Fitness was measured by the number of sigmoidal body bends per worm per minute. IDV concentration = 300μM. Anti-oxidant concentrations = 100μM. Statistics were calculated by a two sided student’s t-test assuming unequal variance: IDV compared to control of that same time point; IDV + anti-oxidant compared to IDV of that same time point; Control 48h vs Control 24h, and Control 72h vs Control 48h. * = P<0.05, ** = P<0.01, *** = P<0.001, n.s. = not significant.

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>IDV</th>
<th>IDV + NAC</th>
<th>IDV + LAA</th>
<th>IDV + ALCAR</th>
<th>IDV + Trolox</th>
</tr>
</thead>
<tbody>
<tr>
<td>24h</td>
<td>129.4 (±13.7)</td>
<td>125.8 (±11.2) **</td>
<td>119.2 (±16.4) n.s.</td>
<td>129.1 (±11.7) n.s.</td>
<td>140.7 (±15.6) ***</td>
<td>132.7 (±11.8) *</td>
</tr>
<tr>
<td>48h</td>
<td>119.5 (±17) ***</td>
<td>106.7 (±22.7) ***</td>
<td>118.2 (±20.2) *</td>
<td>108.1 (±18) n.s.</td>
<td>121.3 (±16.4) **</td>
<td>130.2 (±15.1) ***</td>
</tr>
<tr>
<td>72h</td>
<td>107.7 (±16.3) ***</td>
<td>95.0 (±25.6) ***</td>
<td>96.6 (±23.7) n.s.</td>
<td>105.7 (±15) n.s.</td>
<td>100.7 (±21.9) n.s.</td>
<td>94.7 (±19.2) n.s.</td>
</tr>
</tbody>
</table>

2.10 Changes in gene expression upon exposure to PIs

To probe which pathways react to short-term induced toxicity, a selection of genes involved in the unfolded protein response (UPR), the ubiquitin proteasome system (UPS), the oxidative stress response, and mtDNA transcription and mRNA\textsuperscript{mt} translation, were screened for altered expression using a mRNA qPCR (Figure 10).

Figure 10. Relative fold changes of selected genes after IDV exposure. A: 6h, B: 24h. Statistics were calculated by a two sided student’s t-test assuming unequal variance, compared to unexposed animals (control) of that same time point. * = P<0.05, ** = P<0.01.

Oxidative stress responses can be governed by the the redox sensitive transcription factor SKN-1. SKN-1 is a ROS dependent master regulator of Phase II detoxification enzymes, such as GST-4, and also acts in multiple longevity pathways \textsuperscript{288,289}. skn-1 expression was not effected after 6h (Figure 10A) or 24h (Figure 10B) IDV
HIV-1 protease inhibitors cause immediate mitochondrial dysfunction exposure. To further examine the effects of IDV induced elevated ROS levels we assessed ndx-1 expression. NDX-1 plays a critical role in the removal of oxidized bases from the nucleotide pool before they can be incorporated into DNA.\textsuperscript{417} ndx-1 expression showed a trend towards higher expression after 6h (Figure 10A) and a trend towards lower expression after 24h (Figure 10B) exposure to IDV.

Oxidative damage to MRC proteins and chaperones induces a unique PTEN-induced putative kinase 1 (PINK-1) mediated mitophagy pathway.\textsuperscript{418} Mutations in pink-1 cause enhanced sensitivity to oxidative stress, decreased MRC activity and altered mitochondrial morphology.\textsuperscript{419,420} pink-1 expression levels were significantly lower than controls after 6h (Figure 10A) exposure to IDV, and they showed a rise (which is not significant due to the large variation between biological replicates) after 24h (Figure 10B).

Oxidative stress is known to inhibit mitochondrial protein import, affecting the levels of mature functional proteins in the mitochondria and prompting activation of the UPR.\textsuperscript{366} Mitochondrial heat shock protein 6 (HSP-6) is the C. elegans homologue of HSP-70 in humans and is strongly expressed upon perturbations in mitochondrial protein homeostasis.\textsuperscript{365} Besides hsp-6, we quantified hsp-4 expression. HSP-4 is specific for the endoplasmic reticulum UPR and, if upregulated, would point towards systemic instead of mitochondrial specific responses to PIs.\textsuperscript{421} Moreover, PIs have been found to cause ER stress.\textsuperscript{100} hsp-6 nor hsp-4 expression levels were significantly changed after 6h (Figure 10A) and 24h (Figure 10B) exposure to IDV.

C34B2.6 encodes a homolog of the mitochondrial Lon protease which is crucial for mitochondrial quality control and adaptation to acute mitochondrial oxidative stress by proteolytic removal of oxidized proteins. Lon protease is also involved in protecting mtDNA from oxidative damage and is associated with correct assembly of MRC complexes.\textsuperscript{365,422} After 6h treatment with IDV, C34B2.6 expression remained unchanged (Figure 10A). After 24h, however, C34B2.6 mRNA levels were significantly lower than controls (Figure 10B).

The UPS is the principal mechanism for the degradation of damaged or incorrectly folded proteins that cannot be refolded by chaperones. The UPS functions throughout the entire cell, though mechanisms exist to recruit UPS components to the mitochondria for specific degradation of mitochondrial proteins.\textsuperscript{423} pas-6 encodes a type 1 alpha subunit of the 26S proteasome's 20S catalytic core.\textsuperscript{364} The 20S catalytic core can degrade proteins by two methods; one as part of the UPS, which requires ubiquitin ligases and the 19S regulatory particle, and the other which has a role in the acute stress response to oxidative damage and is 19S and ubiquitination independent.\textsuperscript{424} pas-6 showed a trend towards decreased expression after 6h (Figure 10A) which became significant after 24h (Figure 10B) exposure to IDV.

MtDNA replication was not stalled by IDV treatment (Figure 7). However, mtDNA copy numbers do fluctuate during PI exposure (Table 3 & 4), indicating that mtDNA replication mechanisms are triggered in response to PIs. In an attempt to pinpoint a pathway that regulates PI induced mtDNA replication change, we quantified polg-1 which encodes the mitochondrial DNA polymerase-\textgamma.\textsuperscript{61} Consistent with observations by Davis et al. who demonstrated that the mitochondrial transcription factor A, and not polymerase-\textgamma, is the major regulator of mtDNA replication,\textsuperscript{425} polg-1 expression levels remained unchanged after 6h (Figure 10A) and 24h exposure
Chapter 5

(Figure 10B) to IDV. We also measured expression of mrps-5 which encodes the translation regulator mitochondrial ribosomal protein S-5 (MRPS-5). mrps-5 expression levels were significantly lower than controls at 6h (Figure 10A) and 24h (Figure 10B).

3. Discussion
A favoured theory for PI induced adverse events and mitochondrial toxicity is an excess production of ROS. Although high levels of ROS have been observed after 30 minutes exposure of PIs to human peripheral blood mononuclear cells, few studies have focussed on short-term events and - to date - no clear mode of action has been discovered that explains how PIs cause mitochondrial toxicity. In this study we have endeavored to clarify the extent of mitochondrial dysfunction and increased ROS production in PI induced adverse events. In particular, we have focused on the chronology of events during short-term mitochondrial toxicity.

In summary, we have observed that the PIs used in this study cause rapid MRC dysfunction. This was visualized by a marked decrease in ATP levels after 2.5 minutes (Figure 5A) and a lower oxygen consumption rate after 5 minutes (Figure 5B). Additionally, ROS flux is reduced within 10 minutes of PI exposure (Figure 4), supporting our findings that MRC function is perturbed. We suggest that the cause for this prompt failure in MRC function is due to the increased generation of ROS, as ATP levels are normalized upon supplementation with AOXs (Table 7). The origin and localization of the produced ROS, however, remains elusive. A sharp decreased expression of gst-4 within 1h of exposure suggests that cytosolic ROS are possible suspects (Figure 1). In an attempt to understand the response of C. elegans to acute PI induced mitochondrial toxicity we assessed expression levels of genes involved in various mitochondrial quality control pathways. Although the results need further verification, we discovered possible roles for pink-1, C3B42.6 (lorn-protease), pas-6, and mrps-5 (Figure 10).

In this study we have also investigated the role of mitochondrial toxicity and ROS generation during chronic exposure (>24h) to PIs. sod-3 shows a trend towards increased expression after 72h, which is suggestive of a role for increased mitochondrial ROS generation in PI toxicity after long-term exposure. Additionally, 72h of PI exposure resulted in fragmented mitochondrial networks (Figure 6). 24h, 48h, and 72h treatment resulted in reduced fitness, as measured by thrashing and fecundity assays (Table 5 & Figure 8). We show evidence that the PI induced diminished fitness and mitochondrial network fragmentation is likely not caused by inhibition of mtDNA replication (Figure 7 & Table 3). For some PIs the reduction in thrashing at 24h and 48h could be attenuated by supplementation with AOXs (Table 8), supporting a role for ROS in adverse events associated with chronic exposure.

3.1 The role of ROS in short-term PI induced adverse events
Most studies suggest a mitochondrial origin for PI induced ROS production (Chapter 1). However, the direct increase in ROS levels is likely to be of cytosolic origin, as PI localisation within the mitochondrial matrix has never been shown. Therefore, Reyskens and Essop proposed that PIs diminish cytosolic antioxidant defence systems, triggering an increase in cytosolic ROS. This then raises ROS levels in the inter-mitochondrial
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membrane space causing a perturbation of MRC activity, which in turn, leads to increased mitochondrial ROS generation. In accordance with this, decreased glutathione levels and Cu/ZnSOD activity have been found after 24h exposure of rat pancreatic insulinoma cells (INS-1) to NFV. Our results also support this hypothesis, as we show that within an hour a decrease in gst-4 expression is apparent for all PIs tested. Interestingly, IDV and NFV show similar patterns in gst-4 expression changes and H$_2$O$_2$ production rate, as do RTV and SQV, which indicates that there may be a correlation between these events. Additionally, mitochondrial specific sod-3 expression after 4h exposure to IDV remains unchanged. Although the sod-3 expression results need further verification as very few animals were analyzed, these results indicate that mitochondria are not the initial source of PI induced ROS production. The exact way in which PIs cause a decrease in ROS detoxifying enzyme expression, however, remains to be explained.

To detect ROS and measure ROS flux we have used the fluorescence reporter dye H$_2$DCFDA which is predominantly sensitive to H$_2$O$_2$ and not superoxide. Superoxide is typically produced in the mitochondria at MRC complexes I and III and is rapidly converted to H$_2$O$_2$ and oxygen by mitochondrial SODs. As H$_2$O$_2$ is relatively stable it is able to transfuse across the mitochondrial outer membrane where it can perform signalling functions. We can therefore deduce that ROS flux is diminished upon exposure to PIs (Figure 3 & 4), as this coincides with a decreased metabolism and a lower hydrogen peroxide production rate.

3.2 ROS induced MRC dysfunction

Elevated levels of ROS in the vicinity of the MRC are known to perturb MRC function. Our observations that supplementation with AOX nullifies the rapid decrease in ATP levels supports this (Table 7). Our use of NAC, LAA, ALCAR, and Trolox, to rescue MRC function, signifies that the initial increase in ROS is likely located in the cytosol as these AOX do not preferably accumulate in the mitochondria. The precise type and localization of ROS that cause MRC dysfunction, however, remains to be revealed. We did not see a rise in H$_2$O$_2$ during the first hour of PI exposure which points towards other ROS as possible causative agents. To observe if these ROS are of mitochondrial origin, MitoSOX may prove sufficient 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.

3.3 The mitochondrial response to PI induced MRC dysfunction

The changes seen in mitochondrial MRC function and ROS flux may be responsible for the observed rise in mtDNA copy number, 1h after PI exposure. Endogenous or exogenous oxidative stress, glutathione depletion, or reduced MRC function, has been shown to increase mtDNA copy number and mitochondrial biogenesis in human and animal cells, likely in an attempt to produce more functional mitochondria. In agreement, IDV has been shown to increase mtDNA content in brown adipocytes, which parallels with an increase in cytochrome c oxidase subunit II (COX2) mRNA and COX activity. Compared to RTV and SQV at 1h exposure, IDV and NFV cause a similar amount of mtDNA copy number increase which, interestingly, coincides with their ability to induce similargst-4 expression and ROS flux changes.
We observed that chronic exposure, however, does not induce large fluctuation in mtDNA copy number, even though changes are apparent (Table 3). In one study, decreased expression of the mitochondrial-encoded COX2, but not the nuclear DNA-encoded subunit IV (COX4), both from the MRC complex IV, was observed in fibroblasts after long-term treatment with IDV and NFV. Moreover, a significant decrease in mitochondrial membrane potential and increased ROS production from the mitochondria was found, which suggests that PI induced mitochondrial dysfunction is significant to chronic adverse events. Moreover, protein translation is known to be negatively affected by ROS and we found decreased expression of mrps-5 (Figure 10) which is in accordance with these observations.

3.4 Mitochondrial toxicity from long-term PI exposure

Mitochondria are known to have a central role in ageing and we previously suggested that PI exposure is related to mitochondrial dysfunction mediated premature and accelerated ageing. We have shown that PIs strongly reduce thrashing rates during 24-72h exposure (Table 5) and that progeny number are significantly reduced from exposure to NFV, RTV, and SQV (Figure 8). We found that of the PIs tested; only SQV reduced mean and maximum lifespan significantly compared to its 3% DMSO control (Table 6). RTV showed a trend towards an increased mean and a reduced maximal lifespan, and IDV showed a trend towards a decreased mean lifespan. These results, however, need to be considered with caution as the lifespans in the presence of PIs have not been duplicated. Moreover, DMSO at relevant concentrations also significantly alters lifespan. More research is needed to verify the possibility that PIs cause premature and accelerated ageing. Supplementation of RTV with NAC showed no change in lifespan compared to RTV alone (Table 6). However, as RTV does not significantly change lifespan compared to the control we cannot dismiss a role for ROS in long-term toxicity. The concentration dependent trend towards a rise in sod-3 expression after 72h IDV exposure testifies to this. Additionally, the decrease in mtDNA copy number caused by IDV after 72h exposure could be attenuated by AOX (Figure 9B), which taken together suggests that ROS do influence mitochondria during chronic exposure.

After 72h exposure to PIs, we found that mitochondrial morphology is significantly altered. Specifically, we find increased fragmentation of mitochondrial networks (Figure 6). NFV and RTV induced mitochondrial network fragmentation has been documented before in HeLa cells after 12h and 24h exposure. Variations in cellular bioenergetics, in particular a reduction in ΔΨm, have been shown to rapidly change mitochondrial morphology; in particular swollen mitochondria and a fragmented mitochondrial network. Conversely, altered mitochondrial morphology affects mitochondrial function and extensive mitochondrial fission and fragmentation increases ROS leakage. To elucidate if PI elevated ROS and consequent MRC dysfunction are pivotal in the process of altering mitochondrial morphology it is essential to evaluate mitochondrial morphology after short-term exposure. Chronic exposure to PIs likely hampers many cellular processes and the precise role of ROS herein becomes only more convoluted as exposure time is lengthened (Chapter 1). The unclear results from rescue of fitness with AOX (Table 8), underscores the complexity of PI induced mitochondrial toxicity during chronic exposure. We can conclude, however, that mtDNA replication is not stalled by PIs and is thus not responsible for the observed toxicity during chronic exposure (Figure 7).
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3.5 PIs interfere with many cellular processes

Our findings show that there is not a PI concentration dependent decline in ATP levels for IDV, NFV and RTV (Figure 5A). This may indicate that other processes besides the generation of ROS are also triggered upon exposure to PIs (see Introduction, 3.8). For instance, PIs have been found to have different or even contradictory effects depending on their concentration, and there are indications that increased ROS production only occurs after exposure to a certain threshold concentration of PIs (Chapter 1). In line with this, IDV, NFV, and SQV, have no effect on ATP levels at 200\(\mu\)M, but do at 300\(\mu\)M. Moreover, RTV induced ATP depletion fluctuates depending on the concentration; lower ATP levels are found at 200\(\mu\)M and 500\(\mu\)M than at 300\(\mu\)M, and 200\(\mu\)M has the strongest effect of all the concentrations tested (Figure 5A). An explanation for these results may lie in the fact that PIs are known to modify activity and expression of active drug transport systems which in turn may alter drug absorption, elimination, and tissue distribution (Chapter 1). 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. Further research into the ability of PIs to change pharmacokinetics in C. elegans is warranted and may help shed some light on the use of PIs in combination therapy (see Chapter 6).

3.6 Mitochondrial quality control is affected by PI exposure

In an attempt to understand the cellular response to PI exposure we quantified transcriptional expression of a selection of genes involved in mitochondrial surveillance and quality control. Although the results need further verification, we discovered possible roles for pink-1, C34B2.6 (lon-protease), pas-6, and mrps-5 (Figure 10). Initially, we suspected that skn-1 would play a role in the cellular reaction to IDV, as oxidative stress responses have been found to be strongly governed by the redox sensitive transcription factor SKN-1. SKN-1 is known to respond to ROS, however, by translocating from the cytosol to the nucleus where it regulates gene expression. A direct effect of IDV induced ROS on skn-1 expression may therefore not be apparent, at least not at the time-points tested. For further research, we suggest the use of a skn-1 knockout (zu67) to verify if cellular responses to PI induced ROS are governed by SKN-1.

We also suspected hsp-4 expression to be changed as 40\(\mu\)M PIs have been found to cause ER stress in mouse and various human cell lines after 24h exposure. However, no significant changes were observed in our experiments. This could be explained by the use of different drug concentrations or model systems. Nonetheless, ER stress is known to be a cause and an effect of ROS production, and ROS caused by ER stress are known to derive from MRC dysfunction. The ER and mitochondria are intricately connected; sharing phospholipids and protein degradation machinery, and the ER has been implicated to aid in mitochondrial fission. Taken together, PI induced ER stress, at least after long-term exposure, may be a cause for increased mitochondrially derived ROS and changes in mitochondrial function and morphology. Research into the roles of the ER during PI treatment is needed and may clarify many of the observed effects PIs have on mitochondrial function.
We observed changes in \textit{pink-1} expression levels indicating a role for PINK-1 in the cellular response to PI toxicity. PINK-1 is, for instance, involved in mitophagy\textsuperscript{434} and its possible upregulation after 24h IDV exposure may influence mitochondrial morphology. Interestingly, IDV has been shown to significantly inhibit the mitochondrial processing protease (MPP) in yeast\textsuperscript{118}, and inhibition of MPP causes PINK-1 accumulation on the mitochondrial surface which triggers mitophagy\textsuperscript{435}. Further roles for PINK-1 in PI toxicity may lie in the close association between PINK-1 and the MRC. For example, PINK-1 stabilizes MRC complexes and thus assists in maintaining the integrity of the MRC\textsuperscript{420}. Moreover, PINK-1 has been proposed to regulate complex I activity and thus help sustain $\Delta\psi_m$\textsuperscript{436}.

We also observed significant decreased expression of genes encoding proteins that are involved in pathways responsible for removal or degradation of damaged or incorrectly folded proteins. Interestingly, Lon protease protein levels have been found to be elevated in lipodystrophy patients chronically treated (an average of 72 weeks) with HAART\textsuperscript{137}. We, however, show in \textit{C. elegans} that Lon protease (C34B2.6) is down-regulated after 24h exposure to IDV. We believe that this discrepancy is not necessarily contradictory because single-drug treatment is not comparable to HAART. The down-regulation of Lon protease may even point towards a mechanism through which PIs cause mitochondrial toxicity and increased ROS generation. Lon protease is namely essential for mitochondrial adaptation to acute oxidative stress through proteolytic removal of oxidized proteins\textsuperscript{438}. A decreased expression of Lon protease during short-term exposure may prompt mitochondrial failure, which then later on may trigger an increased expression of Lon protease as an adaptive response.

NFV and SQV are known to perturb proteasome 26S and 20S peptidase activity\textsuperscript{439}. We observed down-regulation of \textit{pas-6} which encodes a type-1 alpha subunit of the 20S proteasome catalytic core\textsuperscript{364}. Coinciding with our findings, Segref \textit{et al.} discovered that inhibition of MRC components and increased ROS production in \textit{C. elegans} resulted in disrupted UPS function in the cytosol\textsuperscript{237}. Taken together, these results may point towards a mechanism through which PIs can induce toxicity. The UPS has been found to interface with mitochondria in multiple ways. For example, mitochondrial morphology dynamics are regulated by the UPS, and the UPS works in close concert with PINK-1 to regulate mitophagy\textsuperscript{434}. Changed UPS activity has been proposed to potentiate the development and progression of metabolic diseases which are common in PI treated patients\textsuperscript{237,400}. We hypothesize that the UPS together with Lon protease play a central role in PI induced toxicity and disruption of their function may underlie mitochondrial toxicity and increased ROS generation.
4. Materials and Methods

4.1 Strains and culture conditions
Nematodes were cultured on NGM plates and fed OP50 Escherichia coli lawn at 20°C unless mentioned otherwise. Nematode strains N2 Bristol (wild type), MJCU017, and CF1553 were provided by the Caenorhabditis Genetics Center (CGC) at the University of Minnesota. gst-4::gfp nematodes were a kind gift from Y. Budovskaya. ROS measurements were performed using ‘WOPS3’ (gst-4:: (unc-119(ed3) III, kls17;gst-4::gfp, pDPM#0168) X); unc-119(ed3)(?);Is(unc-54pro::mCherry;unc119(+)), which was generated using MJCU017.

4.2 Antiretroviral drugs & antioxidants
Antiretroviral drugs (Sigma-Aldrich) were dissolved in dimethyl sulfoxide (DMSO) (IDV, NFV = 100mM; RTV, SQV = 10mM). Antioxidants (Sigma-Aldrich) l-ascorbic acid, N-acetylcysteine, and Acetyl-l-carnitine hydrochloride were dissolved to a stock solution of 500mM in dH2O. Trolox, a water soluble α-tocopherol analogue, was dissolved in dH2O containing 5% DMSO to stocks of 500mM.

4.3 Lifespan assays
Lifespan scoring was conducted at 20°C. ~100 synchronized nematodes were placed on NGM plates containing the compound(s) of interest and dead animals were scored approximately every other day. Worms that crawled off the plates, showed bagging or gonad protrusion were removed from the plates and scored separately. Animals were scored as dead when they no longer moved or showed pharyngeal pumping when they were successively gently prodded on the head and tail with a platinum wire. All experiments using L4 animals were done in the presence of 50μM fluorodeoxyuridine (FUDR) to inhibit progeny. One biological replicate for the PIs, and for controls a minimum of 2 biological replicates was analysed. Prism 6 software was used for statistical analysis using the log-rank (Mantel-Cox and Gehan-Breslow-Wilcoxon) method. Age refers to days following hatching (L1).

4.4 Progeny assay
Synchronized L4 staged wildtype (N2) hermaphrodite nematodes (N≥2) were picked to Escherichia coli OP50 pre-seeded NGM plates with 200μM of PI or without drug exposure (control). Progeny (eggs and L1 larvae) were counted every 24h for 4 days. Adult animals were transferred to a fresh plate every 24h to avoid crowding and to facilitate progeny counting. Brood size was determined at 20°C for at least two biological replicates.

4.5 Thrashing assay
A single worm was placed in a drop of M9 buffer on a clean glass slide and allowed to acclimatize for 30 seconds. The frequency of sigmoidal body bends was counted during 30 seconds as described previously. Thrashes were averaged from 10 worms per treatment condition over three independent trials.
4.6 ROS measurements

2′7’-Dichlorofluorescein oxidation

The level of intracellular general ROS was measured by using the fluorescent dye 2′7’-Dichlorofluorescein oxidation (H$_2$DCFDA; Sigma-Aldrich) according to an adapted protocol of Strayer and coworkers$^{315}$. In short, ~200 synchronized wild type, 2h post L4 stage, nematodes were transferred to 96 well plates containing PIs (300μM). 50μM DCF-DA in Citrate Phosphate Buffer (pH = 6.5) was added to the wells directly prior to measurement. Measurements were performed at 25°C and recorded by a FLUOstar OPTIMA plate reader (Biotek Synergy Mx; excitation 485nm and emission 520nm). For quantification of ROS production rate, samples were read every 10 minutes for 4 hours. For short-term fluorescence measurements, samples were read every 2 minutes. DCF fluorescence was normalized to the number of worms per well. Experiments were performed with a minimum of three biological replicates.

gst-4 expression

Short-term experiments (6h) were performed on therapy naïve WOPS3 animals that express GFP which is under control of the gst-4 promoter. Synchronized worms were cultured at 25°C to L4, transferred to FUDR plates until day 1 of adulthood and washed from the plates with M9 buffer and filtered over a SEFAR NITEX® 31μM pore mesh (03-31/24) to remove debris and E. coli. ~800 animals were placed in each well of a flat clear-bottomed 96 well plate and exposed to 300μM of PIs in Citrate Phosphate Buffer (pH = 6.5), in a total volume of 100μL. Measurements were performed at 25°C and recorded by a FLUOstar OPTIMA plate reader (Biotek Synergy Mx). GFP (470/520nm) expression was normalized to constitutively active RFP (577/620nm) expression. Statistical analysis compared to DMSO: IDV & NFV = 0.3%; RTV & SQV = 3%. Experiments were performed with a minimum of three biological and five technical replicates.

sod-3 expression

CF1553 animals were exposed to IDV (mixed into the NGM) from the L4 larval stage, picked onto 1% agarose pads containing 10mM NaN$_3$, and directly imaged. CF1553 animals express GFP which is under control of the sod-3 promoter. Images of at least 4 live animals were captured using the Zeiss Axiovert 40CFL microscope equipped with an Axiovert digital camera, and analysed using ImageJ software. Mid-body (vulvar) sections of animals were selected for analysis.

4.7 Quantitative real time PCR

mtDNA copy number

Quantitative real time PCR was performed as described by de Boer$^{52}$. In short, synchronized wild type (N2), 2h post L4 moult, young adult worms were transferred to OP50 seeded FUdR NGM plates containing PIs (300μM). Five adult worms were collected at predetermined time points during drug exposure and lysed in Lysis buffer (50mM KC, 10mM Tris (pH 8.3), 2.5mM MgCl$_2$, 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 2μl was used as input in the PCR reaction. Primers specific for cytochrome c oxidase subunit I (COXI) were used for the determination of mtDNA copy number. PCRs were performed using the Taqman® universal cycling conditions
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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. mtDNA quantitative PCR was performed with at least three biological and two technical replicates.

mRNA copy number

Worms were synchronized and hatched overnight in M9 on a shaker at 20°C and grown on NGM plates seeded with E. coli OP50 at 20°C. After reaching the L4 stage, they were transferred to NGM plates containing FUdR and the compounds assessed. At time points 2, 6 and 24 hours after exposure, worms were washed from the plates with M9 buffer, filtered over a SEFAR NITEX® 31μM pore mesh (03-31/24) to remove debris and E. coli, pelleted, and immediately stored at -80°C. RNA was extracted with the Direct-zol™ RNA MiniPrep kit from Zymo Research, using TRIzol, according to the manufacturer’s instructions. RNA concentration was determined using Nanodrop® and quality and purity were assessed with RNA ScreenTape®. Primers were designed using the Applied Biosystems Primer Express™ V2.0 software and, where possible, designed to be intron spanning (Table 9). All primers were checked for specificity by the NCBI Primer Blast website and verified using PCR. qRT-PCR analysis was adapted from Lezzerini & Budovsky 47 using the Power SYBR Green™ RNA-to-Ct 1-Step Kit (Applied Biosystems, Foster City, CA, USA, Part #4389986). mRNA copy numbers were calculated with a standard curve using Vector pD4H1 using mCherry-forward AGGATTTAAGTGGGAACGC and mCherry-reverse GCATAA CAGGTCCATCCAGAG primers. Biological duplicates and technical duplicates of each condition were measured.

Table 9. Genes assessed for the qRT-PCR analysis. Primers are not intron (Intr) spanning unless otherwise stated. Coding sequence ID (CDS ID) as found on www.wormbase.org.

<table>
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<th>Name</th>
<th>CDS ID</th>
<th>Primers (FW Forward, RV Reverse) from 5’-3’</th>
<th>Amplicon Length</th>
</tr>
</thead>
<tbody>
<tr>
<td>polg-1</td>
<td>Y57A10A.15</td>
<td>FW: TCGTCTCGCCGGATACACT  RV: GGTAGACACGTCCACGGAAATC</td>
<td>75 bp</td>
</tr>
<tr>
<td>ndx-1</td>
<td>T28E3.2</td>
<td>FW: ACAAGCAATCCGCTTCAAAAG  RV: TGCGTCTCCGCGGGATTCCG</td>
<td>77+Intr = 351 bp</td>
</tr>
<tr>
<td>pink-1</td>
<td>EDD6.9</td>
<td>FW: TAAGAGGATCAAATCCATTCTACAAACT  RV: ATGACATCTCGTGCCACATAATT</td>
<td>106 bp</td>
</tr>
<tr>
<td>mps-5</td>
<td>T26A10.1</td>
<td>FW: TCACAAAATCTGGCAAACAAGA  RV: CGGAGACGDCACACATTTG</td>
<td>85+Intr = 731 bp</td>
</tr>
<tr>
<td>C34B2.6</td>
<td>C34B2.6</td>
<td>FW: TCGATATCCACTATTTCCAGGATTT  RV: CGCATCCATGCTTCCAGAGAT</td>
<td>108+Intr = 197 bp</td>
</tr>
<tr>
<td>skn-1</td>
<td>T19E7.2b</td>
<td>FW: GACAGTGATACGCGGATTCG  RV: CGATCCGCTGCTTCCAGAGAT</td>
<td>79+Intr = 131 bp</td>
</tr>
<tr>
<td>hsp-6</td>
<td>C17H5.8</td>
<td>FW: CGGATGGGTGTTTGGTAAAG  RV: GAACCTGGCCTGCTGTGGCAT</td>
<td>113 bp</td>
</tr>
<tr>
<td>hsp-4</td>
<td>F4382.8</td>
<td>FW: CAAACAGAAGAAACCGCATTCAACAA  RV: GATCCAACCTCCACCTTACATTGA</td>
<td>79 bp</td>
</tr>
<tr>
<td>pas-6</td>
<td>CD4.6</td>
<td>FW: TCAGAGAAAGATGTACGAAATTGACA  RV: TGGAAGATAGGACGCAAGAT</td>
<td>90 bp</td>
</tr>
</tbody>
</table>

4.8 ATP measurements

ATP luminescence measurements were adapted from Lagido et al. 2008 251. 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. The antioxidants were dissolved in MiliQ, except for Trolox, which was dissolved in DMSO. 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 the 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.

4.9 Oxygen consumption rate

Oxygen consumption was measured using the Seahorse XF96 (Seahorse Bioscience). 20 L4 animals were picked from NGM plates and transferred in 96-well Seahorse plates containing dH2O. 10x concentrated compounds were injected into the wells after 4 basal measurements, which were used for normalisation. Oxygen consumption was measured in 6 replicates. Data was analysed using Wave 2.2.0 (Seahorse Bioscience).

4.10 2DANCE

Cell culture and PI treatment

Human Embryonic Kidney 293 (HEK293T) cells were cultured in DMEM containing 4.5g/l glucose, 2mM L-glutamine, 1mM sodium pyruvate, 50μg/ml uridine and 10% fetal bovine serum, at 37°C in a humidified atmosphere with 8.5% CO2. The medium was replaced every 48 hours and cells split so that ~80% confluency was maintained. To test whether the different compounds stall mtDNA replication, cells were treated for 48h with 200μM IDV. All experiments were performed in at least two independent experiments.

Mitochondrial DNA isolation, two-dimensional agarose gel electrophoresis (2D-AGE) and Southern blotting

Mitochondrial DNA was isolated from mitochondria using Cytochalasin B (Sigma-Aldrich) treatment before cell breakage, followed by differential and sucrose gradient centrifugation steps as previously described. The 2D-AGE analysis was performed with minor alterations as previously described. Briefly, 5μg of total mitochondrial nucleic acids were digested with HincII (Thermo Scientific) according to the manufacturer’s recommendation and separated over a 0.4%, then a 0.95% agarose gel in 1xTBE. Southern blotting was performed using standard procedures and blots probed against 13 638–1 009, using a random primed $^32$P-probe spanning nts 35–611 (NCR) of the human mtDNA. The radioactive signal was quantified using phosphor storage imaging (GE healthcare, BAS-IP MS), screens and Molecular Imager FX (BioRad).

4.11 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 (Appendix). 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
HIV-1 protease inhibitors cause immediate mitochondrial dysfunction

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 pre-processed 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 \(^{232}\). Subsequently, the image was binarized according to a Yen auto-thresholding procedure and the resulting mask was used for analysing shape and intensity metrics of objects larger than a predefined size (>12 pixels) on the original image. In addition, global texture metrics were measured on the original image using the GLCM texture plugin (by Julio Cabrera).

For unsupervised clustering and heatmap generation, 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*area)/(\(\pi*\text{major axis}\)^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:

\[
\text{Entropy} = \sum_{i,j=0}^{N-1} P_{i,j} \left( -\ln P_{i,j} \right), \text{ Contrast} = \sum_{i,j=0}^{N-1} P_{i,j} \left( i - j \right)^2 \text{ and Correlation} = \sum_{i,j=0}^{N-1} P_{i,j} \left( \frac{\left( i - \mu_i \right) \left( i - \mu_j \right)}{\sigma_i^2 \sigma_j^2} \right),\text{ with } P_{i,j}
\]

reflecting values of row i and column j in the GLCM.

Statistical analyses were performed in Microsoft excel 2010\textsuperscript{\textregistered}, 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. K-means cluster analysis was performed on the standardized dataset (z-scores), using Euclidean distance as distance metric and the average value as linkage value for the dendrograms. The results were represented in a two-dimensional clustergram.