Mitochondrial toxicity of indinavir, stavudine and zidovudine involves multiple cellular targets in white and brown adipocytes.

Say Viengchareun, Martine Caron, Martine Auclair, Min-Ji Kim, Paule Frachon, Jacqueline Capeau, Marc Lombès, Anne Lombès

Antiviral therapy 2007;12(6):919-29

Mitochondrial toxicity of indinavir, stavudine and zidovudine involves multiple cellular targets in white and brown adipocytes

Say Viengchareun[1,2], Martine Caron[3,4], Martine Auclair[3,4], Min Ji Kim[3,4,5], Paule Frachon[4,5,6], Jacqueline Capeau[3,4], Marc Lombès[1,2], Anne Lombès[4,5,6*]

[1] Inserm, U693, Faculté de Médecine Paris-Sud, Le Kremlin-Bicêtre Cedex, F-94276, France;
[2] Univ Paris-Sud, Orsay, F-91405, France;
[3] Inserm, U680, Faculté de Médecine Saint-Antoine, Paris, F-75012, France;
[4] Université Pierre et Marie Curie-Paris 6 UPMC-Paris 6, Paris, F-75005, France;
[5] Inserm, U582, Institut de Myologie, Paris, F-75013, France;
[6] AP-HP, Groupe hospitalier Pitié-Salpétrière, Paris, F-75013, France;

*Address of corresponding author: Dr Anne Lombès, Inserm, Unité 582; Institut de Myologie; UPMC; Groupe Hospitalier Pitié-Salpêtrière, AP-HP, 75651 Paris Cedex 13, France.

Telephone: (33)142165735, Fax: (33)142165700; E-mail: a.lombes@myologie.chups.jussieu.fr

Short title: Adipocyte mitochondrial toxicity of antiHIV drugs

Abstract 243 words

Abstract

Objective: To evaluate the mechanisms of mitochondrial toxicity associated with antiretroviral treatment.

Methods: 3T3-F442A white and T37i brown adipocytes were exposed to stavudine (10 μM), zidovudine (1 μM) and indinavir (10 μM), alone or in combination. Adipocytes fat content was measured with Oil Red O staining. Quantification of mRNA levels and of mitochondrial DNA content used PCR-based techniques. Mitochondrial activities were evaluated with respiration, ATP synthesis and spectrophotometric assays. Mitochondrial mass was assessed by the fluorescent probe MitoTracker Red.

Results: In both cell types, all the treatments induced a severe defect of adipogenesis (low lipid content and decreased markers of adipogenic maturation: PPARγ2, aP2 but also UCP1 in brown adipocytes) as well as altered mitochondrial function (decreased respiration rate and increased mitochondrial mass). Drug combination did not give additional toxicity. Brown adipocytes appeared more affected than white adipocytes (lower respiration rate and decreased ATP production). The mechanisms of mitochondrial toxicity differed with the drug and the cell type. Only stavudine induced severe mitochondrial DNA depletion in both cell types. With all the treatments, white adipocytes showed a decrease of the expression of mitochondrial and nuclear DNA-encoded respiratory chain subunits (COX2 and COX4) whereas brown adipocytes maintained normal expression in accordance with their increase of the transcriptional factors of mitochondrial biogenesis NRF1 and PRC, but not PGC1α.

Conclusion: Our results provide evidence for dissociation between mitochondrial activity, transcription and mitochondrial DNA content pointing out the complexity of mitochondrial toxicity, which impacts multiple cellular targets.

Keywords: mitochondria, mitochondrial DNA, mitochondrial biogenesis, brown fat, adipocyte

Introduction

Lipodystrophy is the most prevalent side effect of antiretroviral treatment of human immunodeficiency virus (HIV) infection [1]. Anti-retroviral therapy (ART) against HIV most often involves nucleoside analogues inhibitors of the HIV reverse transcriptase (NRTI) and protease inhibitors (PI). The relative contribution of each class of drugs to the pathogenesis of lipodystrophy is still under discussion. NRTI, in particular the two thymidine analogues stavudine (d4T) and zidovudine (ZDV), appear to be major contributors to lipodystrophy, most notably peripheral lipoatrophy [2-3]. First generation PIs, such as indinavir (IDV), are suspected to be involved in central adiposity, insulin resistance and dyslipidemia [4-6]. The combination of PI and NRTI increases both the incidence and severity of lipodystrophy [7-8]. The pathophysiological mechanisms of lipodystrophy are extremely complex and encompass multiple related processes including alteration of adipocyte differentiation, mitochondrial toxicity, adipocyte apoptosis, local inflammation and dysregulation of adipocytokines production. A variety of changes have been documented in the subcutaneous adipose tissue of HIV-infected lipodystrophic patients, such as loss of lipid content, decreased adipocyte size, apoptotic features and mitochondrial alterations including mitochondrial proliferation and morphologically abnormal mitochondria [2-3]. Molecular alterations of patients' adipose tissue have included severe decrease of adipogenic transcription factors [9-10], increased expression of adipocytokines IL6 and TNFα [10-11], profound reduction of mitochondrial DNA (mtDNA) and altered expression of several factors involved in mitochondrial function [2-12-15]. Despite the presence of mitochondrial proliferation, no increase of transcription factors involved in mitochondrial biogenesis was reported in lipodystrophic adipose tissue. NRF1 [12], which ubiquitously promotes the coordinated transcription of many genes involved in energy production [16], was reported to be expressed at normal levels whereas PPARγ coactivator 1α (PGC1α), which is highly expressed in brown but not in white adipose

tissue [17], was reported to be decreased [10-12]. PRC, a PGC1 related coactivator, which transactivates NRF1-target genes, has not been studied to-date [18]. Lastly, increased expression of mitochondrial uncoupling protein 1 (UCP1), a specific marker of brown adipocytes, has been reported, which implicates brown adipocytes in the lipodystrophic process [12-19].

Deciphering the mechanisms underlying lipodystrophy has been difficult because of the difficulty in obtaining patient samples, the diversity of agents and length of anti-viral treatment, the routine use of combination therapy, the impact of the viral infection itself and the need to assess the differential impact on white and brown adipocytes. Cellular models have therefore been very useful tools to elucidate some of these mechanisms. The 3T3L1 or 3T3-F442A murine cell lines, which recapitulate most of the white adipocyte differentiation program under specific culture conditions, have most often been utilised. Although each drug had a different pattern of effects, NRTI, essentially ZDV or d4T, decreased adipocyte differentiation, were pro-apoptotic, enhanced expression and secretion of IL6, IL1β and TNFα, and induced mitochondrial proliferation and depolarization [20-22]. Some PI such as IDV or nelfinavir were pro-apoptotic and altered adipocyte differentiation [21-25]. Although bringing important results, cellular models have failed to address several points. The mtDNA dependent activities (respiration, ATP synthesis) have not been directly measured despite the possible compensatory effect of the observed proliferation of mitochondria. The effect of drugs has not been assessed concomitantly on adipocyte differentiation and mitochondrial function despite the tight functional link between both cellular aspects [26-27]. Only one analysis has been conducted in brown adipocytes [19]. Despite the fact that NRTI and PI are routinely combined in clinical practice, the effects of these drugs have generally been analyzed separately, even though the combined effects may well differ from those elicited by each class of drugs alone.

To address potential mechanisms of mitochondrial toxicity of antiretroviral drugs, we have undertaken a parallel and comprehensive analysis of mitochondrial function and adipocyte differentiation in white 3T3F442A adipocytes and in T37i adipocytes, a new murine brown adipocyte cell line that we have recently developed and characterized [28-30]. We chose drugs with known adverse effects: either NRTI (ZDV and d4T) or PI (IDV). These drugs were used alone and in combination, at concentration close to their Cmax. Adipocyte differentiation was evaluated by determining the cell lipid content and the expression of major factors of the adipogenic cascade. Mitochondrial toxicity was evaluated with respect to the respiratory chain activity (respiration, ATP synthesis and enzymatic assays), mitochondrial mass, mitochondrial DNA content and expression of mitochondrial biogenetic factors. We provide evidence that both white and brown adipocytes are severely affected by the drugs (PI and NRTI, used alone or in combination), which all impaired differentiation and mitochondrial function. However, these two cell types profoundly differ with respect to their mitochondrial adaptive responses to treatment. The results showed us the multiplicity of cellular targets involved in anti-retroviral toxicity on mitochondrial function.

Materials and Methods

Cell culture and drug exposure

3T3F442A preadipocytes were provided by Pr. H. Green (Boston, MA). They were maintained in Dulbecco's modified Eagle's medium (DMEM) with 100 IU/ml penicillin, 100 µg/ml streptomycin, 14.5 nmol/l biotin and 10% newborn calf serum (Invitrogen, Cergy Pontoise, France). Their differentiation was induced by switching to 10% foetal calf serum and adding 100 nM insulin for 8 days. T37i cells were cultured in DMEM/HAM's F12, 10% foetal calf serum, 2 mM glutamine, 100 IU/ml penicillin, 100 µg/ml streptomycin, 20 mM HEPES (Invitrogen, Cergy Pontoise, France) and were differentiated by supplementation with 2 nM triiodothyronine (Sigma Chemicals CO, St Louis, MO) and 20 nM insulin (Sigma) for 7 days. Passages number 10 to 20 and 6 to 12 were used for T37i and 3T3F442A cells, respectively.

Cells were exposed to drugs during their whole period of differentiation, at concentrations close to their maximum concentration (C_{max}) values: 1 µM zidovudine (ZDV, GlaxoSmithKline, Marly le Roi, France), 10 µM stavudine (d4T, Bristol Meyers Squibb Virology, Princeton, NJ, USA), and 10 µM indinavir (IDV, Merck Sharp & Dohme Laboratories).

Analysis of gene expression

Total RNA was isolated using the RNeasy kit (Qiagen, Valencia, CA) and specific gene expression was quantified by real time PCR carried out on an ABI 7700 Sequence Detector (Applied Biosystems, Foster City, CA). Briefly, 1 µg of total RNA was treated with DNase I Amplification Grade procedure (Invitrogen) and reverse-transcribed with 200 units of reverse transcriptase using the Superscript™ II kit (Invitrogen) and random hexamers according to the manufacturer's recommendations.

1/20th of the reverse transcription reaction was used for real time quantitative PCR using the qPCR^{TM} Mastermix Plus for Sybr^{TM} Green I, 2.5 mM $MgCl_2$, 200 µM deoxynucleoside triphosphates, 1.25 U Hot goldstar DNA polymerase (Eurogentec, Seraing, Belgium) and 300 nM of each primer (listed in Supplemental Table 1). Standards were provided by serial dilutions of linearized pGEMT-easy plasmid (Promega) with the amplicons as inserts. Correlation coefficients were > 0.98 and efficiencies of at least 0.98, in all experiments. Results were expressed as a ratio to 18S rRNA level per ng of total RNA before reverse transcription and related to the expression measured with control cells, arbitrary set at 100%.

Measurement of adipocyte differentiation

Adipocyte differentiation was evaluated by the steady-state levels of the mRNA encoding major markers of adipogenic maturation (aP2, PPARγ2, PGC1α and UCP1) and by the Oil Red O staining, which reveals lipid droplets in the cytoplasm of fully differentiated adipocytes and was performed as described [24]. Schematically, after fixation in 10% formalin and one wash in water, cell monolayers were stained with 0.6% Oil Red O solution in 60% isopropanol, and solubilised in 10% sodium dodecyl sulphate. The amount of Oil red O stain was measured by its optical density at 520 nm.

Assessment of mitochondrial function

Respiration was evaluated by polarography at 37°C in a Clarke electrode (Hansatech, UK). 10^6 cells were added to 100 mM KMes pH 7.4, 5 mM potassium phosphate, 1 mM EGTA, 3 mM EDTA, 1 mg/ml bovine serum albumin, 1 mM ADP and solubilised with 20 µg digitonine. Respiration was initiated with 20 mM succinate and 10 µM rotenone , then stopped with 0.75 mM potassium cyanide (KCN). The respiration rate was obtained by subtraction of the curve slope under KCN from that under succinate and expressed as nanomoles oxygen per minute and 10^6 cells.

ATP production was measured in parallel to polarography using ATP chemiluminescent assay, according to the manufacturer's protocol (Amersham). It was calculated by subtraction of the slope of ATP concentration under KCN from that under succinate and expressed as nanomoles ATP/min and 10^6 cells.

Spectrophotometric assays of cytochrome c oxidase and citrate synthase were performed in whole cell homogenate according to [31-32].

Mitochondrial mass index was evaluated by the staining with 50 nM MitoTracker Red 580 (Molecular Probes, Eugene, Oregon, USA) and 10 ng/ml Hoechst 33258 in DMEM of adipocytes seeded on 96 wells plate as described [20]. Quantification of the fluorescent signals was performed on a fluorescence plate reader (Spectrafluor plus, Tecan-France, Trappes, France) at 630 and 460 nm for MitoTracker and Hoechst, respectively. Mitochondrial mass was evaluated as the ratio of the fluorescent signal of MitoTracker Red to that of Hoechst.

Mitochondrial DNA quantification was performed on total DNA extracted from whole cell homogenates using standard procedures based on SDS-proteinase K digestion and isopropanol precipitation. Mitochondrial DNA (mtDNA) was quantified by real time PCR using COX2 gene on the mtDNA as target gene and, as standard, serial dilutions of linearized pGEMT-easy plasmid with the COX2 fragment as insert. Results were expressed as copy number of mtDNA per pg of total cellular DNA and related to values observed in control cells, arbitrary set at 100%.

The steady-state levels of the mRNA encoding factors involved in mitochondrial activity and/or biogenesis was analysed using real-time quantitative PCR on reverse transcribed total RNA and included COX2: a mtDNA-encoded subunit of the respiratory chain, COX4: a nuclear DNA-encoded subunit of the respiratory chain, NRF1, PGC1α and PRC: transcription factors involved in mitochondrial biogenesis.

Statistical analysis

Results were expressed as means ± SEM of the indicated number of independent experiments. Statistical significance was determined using the non-parametric Mann–Whitney test. The threshold of significance was at $p = 0.05$.

Results

In both white and brown adipocytes, all the treatments induced a severe defect of adipogenesis

Treatment with IDV, d4T, ZDV, or the combination of one NRTI (d4T or ZDV) with IDV induced significant reduction of the adipocyte lipid content as shown by Oil Red O staining in the two adipocyte cell lines (Fig. 1A and 1B). In parallel, expression levels of PPARγ2 and aP2, markers of the adipocyte differentiation state were significantly decreased (Fig. 1C). In addition, UCP1, a unique marker of late brown adipocyte differentiation, was similarly decreased (Fig. 1C).

In both white and brown adipocytes, all the treatments induced defective respiration and increased mitochondrial mass

Under treatment with IDV, d4T, ZDV, or the combination of one NRTI (d4T or ZDV) with IDV, significant reduction of the cellular respiration rate (Fig. 2A) and increase of the cell mitochondrial mass (Fig. 2B) were observed in both adipocyte cell lines thus demonstrating the presence of mitochondrial toxicity. The impact of the combined treatment did not significantly differ from that of the drugs used in isolation.

Brown adipocytes were more sensitive to the antiretroviral drugs

Under all treatments, the two adipocyte cell lines differed with respect to their ATP production, which was maintained in 3T3F442A white adipocytes but decreased in T37i brown adipocytes (Fig. 3A). Furthermore, under exposure to ZDV, brown adipocytes, but not white adipocytes, showed a significant decrease of the maximal velocities of COX and CS activities (Fig. 3B and C). White adipocytes tended to increase these activities under all treatments but the increase was significant only under d4T+IDV. In contrast, under the same d4T+IDV treatment, brown adipocytes significantly decreased their COX activity. Overall, there was a significant difference between the two adipocyte cell types since the white

adipocytes were able to maintain their COX and CS activities and their ATP synthesis despite a decreased respiration rate, in contrast to the brown adipocytes.

Mitochondrial DNA content and expression were dissociated from each other and from the respiratory chain defect.

To further evaluate the mechanisms of the drug induced-mitochondrial respiratory chain defect, we quantified the amount of mtDNA, which has been reported to be decreased in lipodystrophic adipose tissue [2-12-15]. Only d4T, in isolation or combination, induced severe mtDNA depletion in both cell types whereas ZDV induced a significant but mild mtDNA depletion in white but not brown adipocytes (Fig. 4A). Consequently, the respiratory chain defect observed with all the treatments could not be solely related to the decrease of the cellular mtDNA content. Interestingly, in brown adipocytes, IDV exposure induced a significant increase in cellular mtDNA content (Fig. 4A), which paralleled the observed increase of COX activity (see Fig. 3B).

The steady-state level of the mtDNA-encoded COX2 subunit of COX was analysed to assess the transcription of the mtDNA. In 3T3F442A white adipocytes, it roughly followed the mtDNA content, being most profoundly decreased under exposure to d4T (Fig. 4B). This was not the case in T37i brown adipocytes, which maintained or even significantly increased the mtDNA-encoded COX2 mRNA under exposure to d4T (Fig. 4B). Under IDV treatment, brown adipocytes showed significant increase of the COX2 mRNA, which paralleled the increase of the mtDNA itself (see Fig. 4A).

Transcriptional response of mitochondrial biogenesis differed in white and brown adipocytes.

The steady-state level of the nuclear DNA-encoded COX4 structural subunit of COX was then analysed to assess the nuclear transcriptional regulation of mitochondrial biogenesis. It strikingly differed in the two cell types, being decreased under all the treatments in white adipocytes but maintained at normal levels in brown adipocytes. This result suggested an

absent nuclear mitochondrial biogenesis transcriptional response in white adipocytes, which was confirmed by the absence of increase of NRF1 or PRC transcripts, major regulators of mitochondrial biogenesis [16-18] (Fig. 4C). PGC1α [33], another regulator of mitochondrial biogenesis, was not expressed at a significant level in white adipocytes as previously reported [17]. In sharp contrast, in brown adipocytes there was a significant nuclear transcriptional response for increased markers of mitochondrial biogenesis, such as NRF1 (up to 200% increased expression) and PRC (up to 500% increased expression) (Fig. 4C). However, in contrast to NRF1 and PRC, PGC1α expression was severely reduced and therefore paralleled the decrease of PPARγ2, whose expression is known to be regulated by PGC1α [17]. The difference between white and brown adipocytes mitochondrial adaptive responses are summarized in Table 1.

Discussion

For the first time, this study brings an integrated evaluation of the antiretroviral drug impact on mitochondrial function in both brown and white adipocytes by analysing the diverse molecular levels (DNA, RNA and protein) involved. We used prototype drugs such as IDV, d4T and ZDV, alone and in combination, at concentrations close to their C_{max} in patients, and during the whole process of cell differentiation. Despite inherent limitations of cellular models (short duration of treatment, exposure to drugs during adipogenic conversion, immortal cell lines), our main findings are that, with each type of treatment, white and brown adipocytes were similarly and severely affected with respect to their adipogenic maturation and mitochondrial function. In addition, combination of drugs did not induce additional toxicity.

A potential role for brown adipose tissue in lipodystrophy has been suggested by the decrease of PGC1α [10-34] and increase of UCP1 [12], specific markers of brown adipocytes. Our data unambiguously show that brown adipocytes are more sensitive than white adipocytes to antiretroviral drugs, significantly decreasing their mitochondrial ATP production and COX and CS activities. These cells were also instrumental to address the role of PGC1α as a key player that connects adipogenesis and mitochondrial biogenesis [33]. With each treatment, PGC1α expression decreased in parallel to markers of adipogenesis (PPARγ and aP2), in contrast to other mitochondrial biogenetic factors (NRF1 and PRC).

The differential effect of PI and NRTI has been much discussed. As previously reported in patients and cell models [9-10-21-23-25-35], IDV treatment decreased the expression of markers of adipogenesis. We show for the first time that IDV in isolation has a direct effect on mitochondrial function, causing a significant decrease in the respiration rate and an increase of the mitochondrial mass in both white and brown adipocytes. The two cell types however differed with respect to their mitochondrial biogenesis transcriptional response,

which was decreased in white adipocytes and increased in brown adipocytes. Whether that differential impact depends on IDV accumulation in the diverse cell types remains to be assessed. In addition the link between IDV impact on murine adipocytes and PI toxicity in patients has to be directly addressed by the comparison of diverse PI in isolation or in combination with ritonavir.

Our integrated analysis of the different molecular levels (protein, RNA and DNA) of mitochondrial function has shown that each level is an independent target for the antiretroviral drugs toxicity. Dissociation of mtDNA transcription and mtDNA content under NRTI exposure has been also reported in humans: either early transcription alteration without mtDNA depletion in adipose tissue of control subjects [34-36] or normal mtDNA transcripts despite mtDNA depletion in patients' blood monocytes [37]. Dissociation between mtDNA content and mtDNA-dependent activity may also occur as shown by the presence of normal COX activity despite a severe decrease in mtDNA following exposure to d4T in white as well as brown adipocytes. These data make it difficult to propose a central role for mtDNA depletion in HIV-infected patients' lipodystrophy [2-12-15].

Significant mitochondrial proliferation has been observed in patients' adipose tissue, in association with mtDNA depletion and decreased mtDNA transcription [9-12-15]. We observed the same response in mouse white adipocytes, which preserved or even increased their COX activity despite severe mtDNA depletion (under d4T), severe decrease of transcripts of mtDNA-encoded and nuclear DNA-encoded subunits (under all treatments) and absence of transcriptional response of mitochondrial biogenesis (NRF1, PRC). These observations suggest the presence of post-transcriptional compensatory mechanisms. Although mitochondrial proliferation has a compensatory function in contributing to the preservation of mitochondrial activities, it may also have deleterious consequences as observed in genetic mitochondrial myopathies [38].

In conclusion, the present study clearly shows the multiplicity of molecular targets for the mitochondrial toxicity induced by antiretroviral drugs, both PI and NRTI. It demonstrates the necessity to assess the effects on DNA, RNA and protein, as well as consider the specific effects of different drugs on different cell types, in order to understand the toxicity associated with antiretroviral drugs. That understanding is mandatory to propose strategies for the prevention or treatment of the adverse effects associated with mitochondrial toxicity.

Acknowledgments

This work was supported by grants from Inserm, ANRS and SIDACTION. The authors are indebted to BMS and Merck Sharp & Dohme, which provided us with stavudine and IDV. We thank Dr Nigel Clarke for his most useful comments on the manuscript.

References

1. Carr A, Cooper DA. Adverse effects of antiretroviral therapy. *The Lancet* 2000;**356**(9239):1423-1430.

2. Walker UA, Bickel M, Lutke Volksbeck SI, et al. Evidence of nucleoside analogue reverse transcriptase inhibitor-associated genetic and structural defects of mitochondria in adipose tissue of HIV-infected patients. *J Acquir Immune Defic Syndr* 2002;**29**(2):117-121.

3. Nolan D, Hammond E, James I, McKinnon E, Mallal S. Contribution of nucleoside-analogue reverse transcriptase inhibitor therapy to lipoatrophy from the population to the cellular level. *Antivir Ther* 2003;**8**(6):617-626.

4. Carr A, Samaras K, Burton S, et al. A syndrome of peripheral lipodystrophy, hyperlipidaemia and insulin resistance in patients receiving HIV protease inhibitors. *AIDS* 1998;**12**(7):F51-58.

5. Carr A, Samaras K, Chisholm DJ, Cooper DA. Pathogenesis of HIV-1-protease inhibitor-associated peripheral lipodystrophy, hyperlipidaemia, and insulin resistance. *The Lancet* 1998;**351**(9119):1881-1883.

6. Miller KD, Jones E, Yanovski JA, Shankar R, Feuerstein I, Falloon J. Visceral abdominal-fat accumulation associated with use of indinavir. *The Lancet* 1998;**351**(9106):871-875.

7. Mallal SA, John M, Moore CB, James IR, McKinnon EJ. Contribution of nucleoside analogue reverse transcriptase inhibitors to subcutaneous fat wasting in patients with HIV infection. *AIDS* 2000;**14**(10):1309-1316.

8. van der Valk M, Gisolf EH, Reiss P, et al. Increased risk of lipodystrophy when nucleoside analogue reverse transcriptase inhibitors are included with protease inhibitors in the treatment of HIV-1 infection. *AIDS* 2001;**15**(7):847-855.

9. Bastard JP, Caron M, Vidal H, et al. Association between altered expression of adipogenic factor SREBP1 in lipoatrophic adipose tissue from HIV-1-infected patients and abnormal adipocyte differentiation and insulin resistance. *The Lancet* 2002;**359**(9311):1026-1031.

10. Kannisto K, Sutinen J, Korsheninnikova E, et al. Expression of adipogenic transcription factors, peroxisome proliferator-activated receptor gamma co-activator 1, IL-6 and CD45 in subcutaneous adipose tissue in lipodystrophy associated with highly active antiretroviral therapy. *AIDS* 2003;**17**(12):1753-1762.

11. Lihn AS, Richelsen B, Pedersen SB, et al. Increased expression of TNF-alpha, IL-6, and IL-8 in HALS: implications for reduced adiponectin expression and plasma levels. *Am J Physiol Endocrinol Metab* 2003;**285**(5):E1072-1080.

12. Pace CS, Martin AM, Hammond EL, Mamotte CD, Nolan DA, Mallal SA. Mitochondrial proliferation, DNA depletion and adipocyte differentiation in subcutaneous adipose tissue of HIV-positive HAART recipients. *Antivir Ther* 2003;**8**(4):323-331.

13. Shikuma CM, Hu N, Milne C, et al. Mitochondrial DNA decrease in subcutaneous adipose tissue of HIV- infected individuals with peripheral lipoatrophy. *AIDS* 2001;**15**(14):1801-1809.

14. Cherry CL, Gahan ME, McArthur JC, Lewin SR, Hoy JF, Wesselingh SL. Exposure to dideoxynucleosides is reflected in lowered mitochondrial DNA in subcutaneous fat. *J Acquir Immune Defic Syndr* 2002;**30**(3):271-277.

15. Nolan D, Hammond E, Martin A, et al. Mitochondrial DNA depletion and morphologic changes in adipocytes associated with nucleoside reverse transcriptase inhibitor therapy. *AIDS* 2003;**17**(9):1329-1338.

16. Scarpulla RC. Nuclear activators and coactivators in mammalian mitochondrial biogenesis. *Biochim Biophys Acta* 2002;**1576**(1-2):1-14.

17. Puigserver P, Wu Z, Park CW, Graves R, Wright M, Spiegelman BM. A cold-inducible coactivator of nuclear receptors linked to adaptative thermogenesis. *Cell* 1998;**92**:829-839.

18. Andersson U, Scarpulla RC. Pgc-1-related coactivator, a novel, serum-inducible coactivator of nuclear respiratory factor 1-dependent transcription in mammalian cells. *Mol Cell Biol* 2001;**21**(11):3738-3749.

19. Rodriguez de la Concepcion M, Domingo JC, Domingo P, Giralt M, Villarroya F. Uncoupling protein 1 gene expression implicates brown adipocytes in highly active antiretroviral therapy- associated lipomatosis. *AIDS* 2004;**18**:959-960.

20. Caron M, Auclair M, Lagathu C, et al. The HIV-1 nucleoside reverse transcriptase inhibitors stavudine and zidovudine alter adipocytes function in vitro. *AIDS* 2004;**18**:2127-2136.

21. Lagathu C, Bastard JP, Auclair M, et al. Antiretroviral drugs with adverse effects on adipocyte lipid metabolism and survival alter the expression and secretion of proinflammatory cytokines and adiponectin in vitro. *Antivir Ther* 2004;**9**(6):911-920.

22. Lagathu C, Kim M, Maachi M, et al. HIV antiretroviral treatment alters adipokine expression and insulin sensitivity of adipose tissue in vitro and in vivo. *Biochimie* 2005;**87**(1):65-71.

23. Zhang B, MacNaul K, Szalkowski D, Li Z, Berger J, Moller DE. Inhibition of adipocyte differentiation by HIV protease inhibitors. *J Clin Endocrinol Metab* 1999;**84**(11):4274-4277.

24. Caron M, Auclair M, Vigouroux C, Glorian M, Forest C, Capeau J. The HIV protease inhibitor indinavir impairs sterol regulatory element- binding protein-1 intranuclear localization, inhibits preadipocyte differentiation, and induces insulin resistance. *Diabetes* 2001;**50**(6):1378-1388.

25. Caron M, Auclair M, Sterlingot H, Kornprobst M, Capeau J. Some HIV protease inhibitors alter lamin A/C maturation and stability, SREBP-1 nuclear localization and adipocyte differentiation. *AIDS* 2003;**17**(17):2437-2444.

26. Roche R, Poizot-Martin I, Yazidi CM, et al. Effects of antiretroviral drug combinations on the differentiation of adipocytes. *AIDS* 2002;**16**(1):13-20.

27. Wilson-Fritch L, Burkart A, Bell G, et al. Mitochondrial biogenesis and remodeling during adipogenesis and in response to the insulin sensitizer rosiglitazone. *Mol Cell Biol* 2003;**23**(3):1085-1094.

28. Zennaro MC, Le Menuet D, Viengchareun S, Walker F, Ricquier D, Lombès M. Hibernoma development in transgenic mice identifies brown adipose tissue as a novel target of aldosterone action. *J Clin Invest* 1998;**101**(6):1254-1260.

29. Buyse M, Viengchareun S, Bado A, Lombès M. Insulin and glucocorticoids differentially regulate leptin transcription and secretion in brown adipocytes. *Faseb J* 2001;**15**(8):1357-1366.

30. Viengchareun S, Zennaro MC, Pascual-Le Tallec L, Lombès M. Brown adipocytes are novel sites of expression and regulation of adiponectin and resistin. *FEBS Lett* 2002;**532**(3):345-350.

31. Srere PA. Citrate synthase. *Meth.Enzymol.* 1969;**13**:3-11.

32. Wharton DC, Tzagoloff A. Cytochrome oxidase from beef heart mitochondria. *Meth.Enzymol.* 1967;**10**:245-250.

33. Puigserver P, Spiegelman BM. Peroxisome proliferator-activated receptor-gamma coactivator 1 alpha (PGC-1 alpha): transcriptional coactivator and metabolic regulator. *Endocr Rev* 2003;**24**(1):78-90.

34. Mallon PW, Unemori P, Sedwell R, et al. In Vivo, Nucleoside Reverse-Transcriptase Inhibitors Alter Expression of Both Mitochondrial and Lipid Metabolism Genes in the Absence of Depletion of Mitochondrial DNA. *J Infect Dis* 2005;**191**(10):1686-1696.

35. Dowell P, Flexner C, Kwiterovich PO, Lane MD. Suppression of preadipocyte differentiation and promotion of adipocyte death by HIV protease inhibitors. *J Biol Chem* 2000;**275**(52):41325-41332.

36. Galluzzi L, Pinti M, Guaraldi G, et al. Altered mitochondrial mRNA production in adipocytes from HIV-infected individuals with lipodystrophy. *Antivir Ther* 2005;**10**(suppl 2):M91-M99.

37. Miro O, Lopez S, Rodriguez de la Concepcion M, et al. Upregulatory Mechanisms Compensate for Mitochondrial DNA Depletion in Asymptomatic Individuals Receiving Stavudine Plus Didanosine. *J Acquir Immune Defic Syndr* 2004;**37**(5):1550-1555.

38. Auré K, Fayet G, Leroy JP, Lacène E, Romero NB, Lombès A. Apoptosis in mitochondrial myopathies is linked to mitochondrial proliferation. *Brain* 2006;**129**(Pt 5):1249-1259.

Table 1: Major mitochondrial alterations of white and brown adipocytes upon exposure to antiretroviral drugs

	White adipocytes (3T3F442A)	**Brown adipocytes (T37i)**
Mitochondrial mass	Increased	Increased
CS activity	Normal (Increased/d4T+IDV)	Normal (Decreased/ZDV or /ZDV+IDV)
OXPHOS activity Respiration rate ATP synthesis COX activity	 Decreased Normal Normal (Increased/d4T+IDV)	 Decreased Decreased Normal (Increased/IDV; Decreased/ZDV or/ZDV+IDV)
mtDNA content	Normal (Decreased/d4T or/d4T+IDV or/ZDV)	Normal (Decreased/d4T or /d4T+IDV; Increased/IDV)
mtDNA transcription (COX2 mRNA)	Decreased (Normal/ZDV+IDV)	Normal (Increased/IDV or /d4T+IDV)
Transcription regulation/ mitochondrial biogenesis COX4 mRNA NRF1 mRNA PRC mRNA PGC1α mRNA	 Decreased Decreased (Normal/d4T) Normal (Decreased/IDV) -	 Normal Increased (Normal/IDV or /ZDV+IDV) Increased Decreased (Normal/IDV)

Only significant changes are indicated. Most alterations were common to several treatments. Alterations specific to certain drugs are indicated between brackets. ATP synthesis is the ATP produced by the mitochondrial oxidation phosphorylation pathway. CS= citrate synthase,

OXPHOS: oxidative phosphorylation pathway, the activity of which was assessed by the rates of respiration and ATP synthesis as well as the cytochrome c oxidase (COX) activity. MtDNA: mitochondrial DNA. Transcription regulation/mitochondrial biogenesis = transcriptional regulation of mitochondrial biogenesis, which was evaluated by the steady state level of the mRNA of COX4, a nuclear DNA-encoded subunit of the respiratory chain, which is under the control of transcription factors involved in mitochondrial biogenesis such as NRF1, PRC and PGC1α.

Figure legends

Figure 1: Altered differentiation of 3T3F442A and T37i adipocytes treated with IDV, d4T or ZDV

3T3F442A (Fig. 1A upper line) and T37i adipocytes (Fig. 1A lower line) were seeded on glass coverslips and submitted to differentiation as described in the Materiel and Methods section. Both adipocyte cell lines were exposed to drugs from the seeding day to the end of differentiation. Drugs were used, alone or in combination, at the following concentrations: 10 µM IDV, 10 µM d4T, and 1 µM ZDV. Oil Red O staining was performed at the end of differentiation in order to measure the amount of lipid accumulation as a marker of adipocyte differentiation. Representative results of staining are shown in Fig. 1A. Quantification of the Oil O Red staining was performed by solubilization of the Oil Red O stained-cells in 10% SDS and measurement of the optical density at 520 nm (Fig 1B). Expression of PPARγ2 , aP2 and UCP1, markers of adipocyte differentiation (Fig. 1C), were assessed by quantitative real time PCR. Results are mean ± SEM of six independent experiments and are expressed as relative values to control cells, which were arbitrarily set at 100%. Statistical significance: * $p>0.05$, ** $p<0.01$ and *** $p< 0.001$, compared to untreated cells (C).

Figure 2: Alteration of mitochondrial function and increase of mitochondrial mass in 3T3F442A and T37i adipocytes under antiretroviral drug exposure

3T3F442A and T37i adipocytes were plated in 100 mm Petri dishes, and exposed to drugs from the seeding day to the end of differentiation. Drugs were used, alone or in combination, at the following concentrations: 10 µM IDV, 10 µM d4T, and 1 µM ZDV. Respiration on succinate (VO_2, Fig. 2A) was determined at day 8 of differentiation. Statistical significance was calculated using the Mann–Whitney U test. * indicates $P<0.05$ as compared to control cells (C). Both cell lines showed a significant decrease of their respiration rate.

Mitochondrial mass index (Fig. 2B) was calculated as the ratio of MitoTracker Red to Hoechst fluorescent signals in cells plated on 96 well plate. Measurements were performed in duplicate in at least 8 experiments. Statistical significance: * $p<0.05$, ** $p<0.01$ and *** $p< 0.001$, as compared to control cells (C).

Figure 3: Distinct mitochondrial enzymatic defects in white and brown adipocyte cell lines treated with antiretroviral drugs

3T3F442A and T37i adipocytes were plated in 100 mm Petri dishes and exposed to drugs from the seeding day to the end of differentiation. Drugs were used, alone or in combination, at the following concentrations: 10 µM IDV, 10 µM d4T, and 1 µM ZDV. ATP synthase activity (ATP, Fig. 3A), cytochrome c oxidase activity (COX, Fig. 3B) and citrate synthase activity (CS, Fig. 3C) were determined at day 8 of differentiation. Statistical significance was calculated using the Mann–Whitney U test : * indicates $p<0.05$ as compared to control cells (C). Brown adipocytes appeared more sensitive to drugs than white adipocytes.

Figure 4: Molecular mechanisms of the mitochondrial toxicity induced by antiretroviral drugs strikingly differ with drug and cell type.

3T3F442A and T37i adipocytes were plated in 6 well-plates and submitted to differentiation in the absence (C) or in the presence of drugs (10 µM IDV, 10 µM d4T, and 1 µM ZDV, used alone or in combination. At day 8 of the differentiation process, total DNA was extracted and mtDNA was quantified by quantitative PCR and expressed as percentage of the mtDNA content in control cells (mtDNA, Fig. 4A). Measurements were performed in duplicate in at least 3 experiments. The average amount of mtDNA in control cells was 473 ±32 copies per pg cellular DNA in 3T3F442A adipocytes whereas it was 745 ± 84 copies per pg cellular DNA in T37i brown adipocytes. Total RNA was extracted from both adipocyte cell lines and reverse-transcribed. The steady-state levels of mRNA encoding for COX2 and COX4, subunits of the mitochondrial respiratory chain (Fig. 4B), as well as NRF1, PGC1α, and PRC,

transcription factors involved in mitochondrial biogenesis (Fig. 4C) were assessed by quantitative real time PCR. Results are mean ± SEM of at least six independent determinations performed in duplicate and normalized by the expression of the 18S rRNA gene. Results are expressed as relative to control cells, arbitrarily set at 100%. Statistical significance: * $p<0.05$, ** $p<0.01$ and *** $p< 0.001$, as compared to control cells.