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Mitochondria are sensors for HIV drugs

Frédéric Petit[1], Bernard Fromenty[2], Andrew Owen[3] and Jérôme Estaquier[1]

[1]Unité de Physiopathologie des Infections Lentivirales, Institut Pasteur, 28 rue du Dr Roux, 75724 Paris cedex 15, France
[2]INSERM U481, Faculté de Médecine Xavier Bichat, 16 rue Henri Huchard BP416, 75870 Paris cedex 18, France
[3]Department of Pharmacology and Therapeutics, 70 Pembroke Place, University of Liverpool, Liverpool L69 3GF, UK

Highly active anti-retroviral therapy (HAART) has drastically altered the course of HIV-1 infection, resulting in a major decrease in morbidity and mortality. However, adverse drug reactions and long-term toxicities associated with HAART are now a concern. A major toxicity that has been highlighted by the increased use of HAART is related to mitochondrial side-effects. At the same time, analysis of the biochemical pathways involved in programmed cell death has revealed that mitochondria are main sensors in this process. In this article, the regulation of mitochondrial damage following the use of nucleoside analogue reverse transcriptase inhibitors (NRTIs) and protease inhibitors is discussed, with a particular focus on the putative molecular mechanisms involved.

HIV-1 and its current therapy

The human immunodeficiency virus type 1 (HIV-1) is a retrovirus and is the causative agent of AIDS. The depletion of $CD4^+$ T cells is a major determinant of HIV-1 pathogenicity [1]. Once HIV-1 has entered a cell, HIV reverse transcriptase converts the single-stranded viral RNA into double-stranded DNA. The double-stranded DNA is then integrated into the host cell genome and transcribed into a full-length mRNA, one of the primary translation products of which is the gag–pol polyprotein product. Proteolytic cleavage of the gag–pol polyproteins by HIV-1 encoded aspartyl protease is necessary for the production of mature infectious virions. Currently licensed anti-retroviral therapies focus on the HIV-1 reverse transcriptase and the HIV-1 protease as potential targets (Table 1) [2]. Nucleoside reverse transcriptase inhibitors [NRTIs (nucleoside analogues)] cause termination of the formation of the DNA chain, whereas non-nucleoside reverse transcriptase inhibitors (NNRTIs) bind directly to, and inhibit, the action of the reverse transcriptase. However, anti-retroviral therapy directed at only the reverse transcriptase of HIV-1 has had limited success because of drug toxicity and the emergence of viral resistance. Protease inhibitors inactivate the HIV-1 protease and prevent the cleavage of gag–pol proteins. Protease inhibitors, unlike reverse transcriptase inhibitors, exhibit their effect late in the HIV replication cycle and are active against chronically infected cells. Thus, combination anti-retroviral therapy of at least two NRTIs and a protease inhibitor is considered to be the standard of care of HIV-1 infected individuals. However, drug toxicity is one of the main obstacles to long-term therapy for HIV-1 infection [3]. Adverse effects of drugs can occur early (within the first 3–6 months of therapy) or late (occurring in individuals who are established on, and tolerating, the drug treatment for some time). Most early toxicities such as nausea, diarrhoea, rash and sleep disturbances are predictable, transient and of mild-to-moderate intensity. Specifically, the lipodystrophy syndrome (LDS) consisting of dyslipidemia, metabolic abnormalities of insulin resistance and redistribution of body fat has become a central issue in the primary care of HIV-infected patients [4,5].

Preventive effects of HIV drugs on apoptosis and loss of mitochondrial membrane potential

Highly active anti-retroviral therapy [HAART (a mixture of NRTIs and protease inhibitors)] aims to slow the rate of viral replication sufficiently to reduce the viral load and thereby stem the emergence of resistant forms of the virus. Thus, HAART produces a significant immune system reconstitution (with sustained increases in circulating levels of $CD4^+$ T cells) after a rapid drop in plasma levels of viral RNA and decreased apoptosis (Box 1). A correlation between the magnitude of apoptosis observed in HIV-infected individuals and the stage of HIV disease has been described [1,6,7]. However, protease inhibitors in addition to exerting anti-viral effects can also have a direct effect on immune cells. Indeed, the susceptibility of peripheral blood T cells to apoptosis is rapidly decreased 4 days following the initiation of treatment with protease inhibitors in adults and children receiving HAART [7]. Therefore, protease inhibitors have clinical and immunological benefits (even in the absence of sustained viral suppression) that might be attributable to anti-apoptotic properties. Several mechanisms might explain how protease inhibitors decrease apoptosis. Protease inhibitors such as ritonavir modulate proteasome activity and major histocompatibility complex (MHC) class I-restricted presentation [8], which might decrease immune activation and

Table 1. Currently licensed anti-retroviral drugs

NRTIs	NNRTIs	Protease inhibitors
Tenofovir	Efavirenz	Indinavir
Emtricitabine	Nevirapine	Saquinavir
Didanosine (ddI)	Delavirdine	Ritonavir
Stavudine (d4T)		Atazanavir
Lamivudine (3TC)		Amprenavir
Zidovudine (AZT)		Lopinavir
Abacadir (ABC)		
Zalcitabine (ddc)		

Corresponding author: Estaquier, J. (jestaqui@pasteur.fr).
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Box 1. Apoptosis pathways

Programmed cell death (PCD) and its main phenotype apoptosis is a cell suicide programme that is essential for development and for adult tissue homeostasis in all metazoan animals. Defects (inhibition or exacerbation) in PCD are involved in several pathologies such as neurodegenerative diseases, cancers or AIDS. The stereotypical death throes of a cell undergoing apoptosis include DNA fragmentation, nuclear condensation, cell shrinkage, blebbing and phosphatidylserine externalization, all of which promote the physiologically silent removal of the cell by its phagocytic neighbours. Mitochondria are implicated in the two major apoptotic pathways currently accepted as the models for cell death. The death receptor-mediated pathway (extrinsic pathway) leads to activation of caspase-8, which cleaves the pro-apoptotic Bcl-2 family member Bid, generating a truncated Bid (tBid). tBid translocates to the mitochondria where it acts with the pro-apoptotic Bcl-2 family members Bax and Bak. Cellular deprival and stress-mediated apoptosis is regulated predominantly at the mitochondrial level (intrinsic pathway). In both pathways, mitochondrial injury is manifested by the release of apoptogenic factors, leading to the apoptotic phenotype [1] (Figure I).

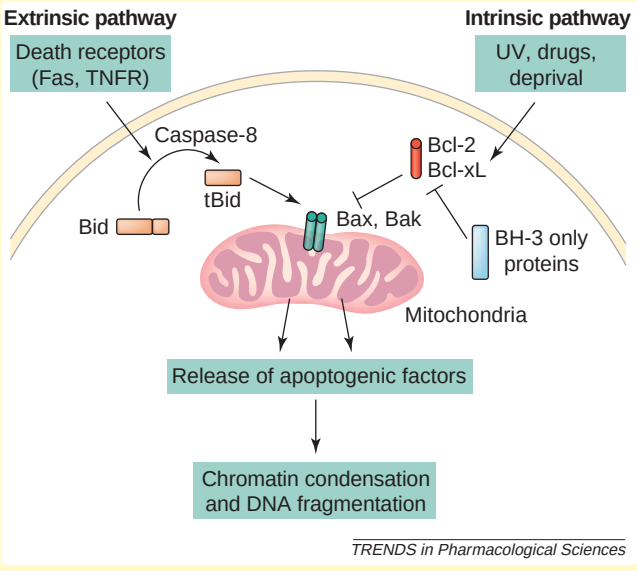

Figure I. Extrinsic and intrinsic pathways involved in apoptosis. Abbreviation: TNFR, tumour necrosis factor receptor.

thereafter decrease activation-induced cell death (AICD). It has also been reported that ritonavir prevents apoptosis and caspase-1 expression in cultured $CD4^+$ T cells isolated from healthy volunteers and HIV-infected individuals [9–11]. However, other reports suggest that the inhibition of apoptotic cell death is not related to alterations in the mRNA of pro- and anti-apoptotic factors, protein synthesis or caspase-1, -3, -6, -7 or -8 activity [12,13]. It has been shown that HIV-infected individuals display both loss of mitochondrial membrane potential ($\Delta\psi m$) [14] and an increase in reactive oxygen species (ROS) production [15]. Moreover, in the lymph nodes of HIV-infected individuals, cell death occurs predominantly following severe mitochondrial damage [16]. To date, the fact that loss in mitochondrial membrane potential is a crucial event in the process of T-cell death during AIDS is well documented [17]. It is interesting to note that following apoptotic stimuli, protease inhibitors can also prevent loss of mitochondrial membrane potential in both intact cells and isolated mitochondria treated with the permeability transition pore complex (PTPC) inducers atractyloside and Vpr [18]. Therefore, protease inhibitors might prevent *in vivo* mitochondrial injury, leading to the restoration of $CD4^+$ T cells.

Side-effects of anti-retroviral drugs

The efficacy and toxicity of any given NRTI analogue is the result of many factors, including protein binding, transport (influx or efflux), and metabolic activation, incorporation and degradation. Patients treated with one or more NRTI analogue experience a variety of side-effects, including peripheral neuropathy, cardiac and skeletal muscle myopathy, pancreatitis, hepatic steatosis, lactic acidosis and bone marrow suppression [4,5,19]. Indeed, dose-dependent peripheral neuropathy is the major treatment-limiting adverse effect of NRTI drugs. Peripheral neuropathy occurs in 34% of patients receiving NRTIs such as zalcitabine (ddC) and is manifested ~6-8 weeks after starting therapy, often as the first indicator of toxicity. However, it is usually reversed following the withdrawal of the offending NRTIs [4,5]. Steatosis and non-alcoholic steatohepatitis (NASH) can also be induced by some NRTIs and provoke lactic acidosis, particularly when zidovudine (AZT) is given as a high-dose monotherapy [4] or in patients receiving stavudine (d4T) and/or didanosine (ddI) [20]. Discordantly low $CD4^+$ T-cell restoration has been reported in HIV-1-infected patients with low viral loads in response to HAART. It was hypothesized that AZT might limit CD4 restoration by a haematotoxic mechanism and a pilot study illustrated that two patients switching from AZT to stavudine had reduced apoptosis and increased $CD4^+$ T-cell counts [21].

Many of the aforementioned drug complications are thought to be attributable to toxic effects on mitochondria, and the effects of NRTI-induced mitochondrial toxicity are known to be tissue specific [4]. Direct examination of subcutaneous adipose tissue is likely to reveal the most conclusive information about the role of mitochondrial toxicity in the development of lipodystrophy syndrome. Cardiomyocyte apoptosis, which can be induced by myocardial cell stretch, oxygen radicals and cytokines (such as tumour necrosis factor), has also been observed during HIV infection. Cardiomyocytes have been reported to die through mitochondrial pathways and death-receptor pathways. Skeletal and cardiac myopathy has been reported predominantly following AZT treatment, but it is clearly dose dependent and low doses of AZT have markedly reduced the occurrence of myopathy [4,20]. Thus, AZT causes a cumulative mitochondrial skeletal myopathy in adult AIDS patients [22]; mitochondria are enlarged and ultrastructurally swollen, and contain disrupted cristae and occasional paracrystalline inclusions [23–26].

Anti-retroviral prevention of mother-to-child transmission of HIV-1 is now well established. However, until recently, evidence of mitochondrial dysfunction was not observed in infants from mothers infected by HIV-1 and exposed perinatally to NRTIs. This is most probably because most drug-exposed HIV-1-uninfected children are asymptomatic [27,28]. Indeed, the deaths of two

HIV-1-uninfected NRTI-exposed children with severe mitochondrial toxicity, and 18 other children with mitochondrial dysfunction, all of whom were exposed *in utero* to AZT or AZT plus lamivudine (3TC), have been reported [29]. In addition, it has also been reported that a significant depletion of mitochondrial DNA (mtDNA) occurs in umbilical cord blood of infants and peripheral blood of 1- and 2-year-old children whose HIV-1-infected mothers received AZT during pregnancy [30–32]. Mitochondrial DNA depletion was also observed in umbilical cord blood and umbilical cord from infants of women receiving combined AZT and 3TC [33]. Recently, another study demonstrated that foetuses of pregnant *Erythrocebus patas* monkey dams given a dosing protocol of AZT with 3TC that was equivalent to that used in humans sustained significant mitochondrial damage in heart, skeletal muscle and brain (cerebrum and cerebellum), compared with unexposed controls [34]. Thus, perinatally exposed HIV-1-uninfected infants might also be at risk of these toxic consequences.

Although less documented, NNRTIs might also act on mitochondria, thereby inducing cell death. Indeed, efavirenz has been reported to induce apoptosis, and impairs the proliferation of T-cell lines and primary T cells of healthy donors [35].

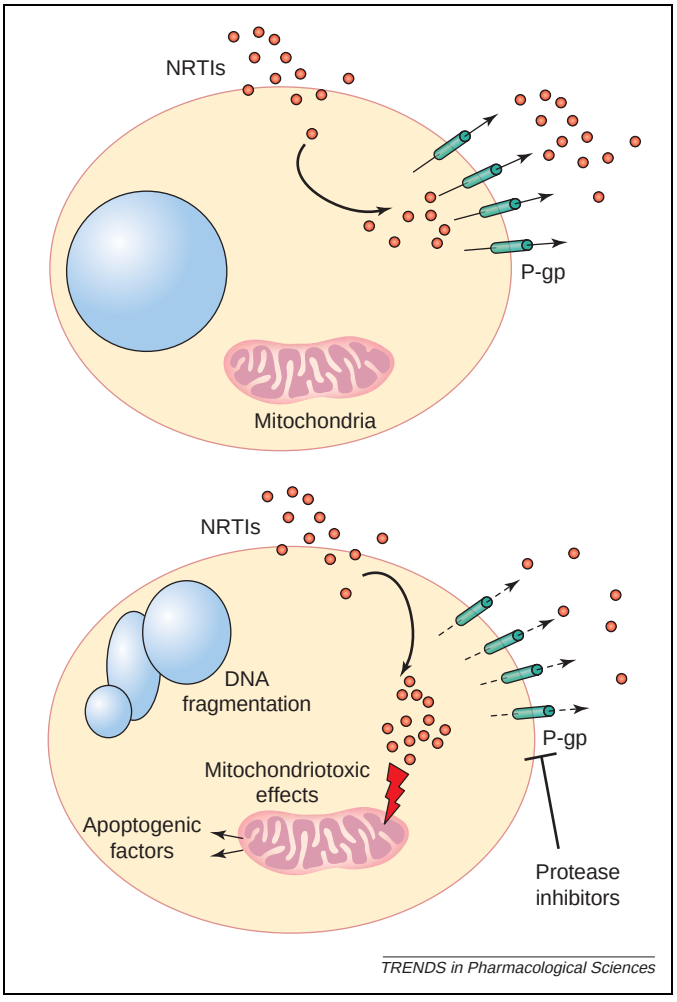

Figure 1. The involvement of drug transporters in the effects of anti-retroviral drugs on cell survival. ABC transporters [e.g. P-glycoprotein (P-gp)], situated on the plasma membrane of macrophages, $CD4^+$ T cells, brain cells and testis cells, transport drugs (e.g. NRTIs) out of the cell in an ATP-dependent fashion. Protease inhibitors inhibit P-gp, resulting in an accumulation of NRTI drugs in the cell, and mitochondriotoxic effects, leading to the release of apoptogenic factors, DNA fragmentation and apoptosis.

Side-effects of protease inhibitors

Whereas much is known about NRTI toxicity, comparably little is known about the side-effects of protease inhibitors. The loss of subcutaneous adipose tissue can result from enhanced adipocyte apoptosis as observed in biopsy samples taken from lipoatrophic areas of patients with phosphatidylinositol-associated lipodystrophy [36,37]. Treatment with protease inhibitors can induce apoptosis of adipocytes by binding cytoplasmic retinoic acid-binding protein 1 (CRABP-1), which is involved in adipocyte differentiation [38]. Recently, it was shown that *in vitro* treatment of healthy donor peripheral blood mononuclear cells (PBMCs) with increasing concentrations of indinavir and saquinavir significantly decreased T-cell proliferation [39,40]. Both indinavir and saquinavir induced a loss of mitochondrial membrane potential at 10 μM but not at 1.0 or 0.2 μM [39]. At 10 μM, saquinavir and indinavir showed toxicity in monocytes and $CD4^+$ T cells, with greater toxicity in the former cells and no effect on $CD8^+$ T cells or the CEM T-cell line. However, this form of cell death was not associated with a chromatin condensation and fragmentation, a defining feature of apoptosis (J. Estaquier, unpublished). Moreover, it has been reported that a near clinical plasma level of ritonavir can directly cause endothelial cell death [41]. Similarly, it was reported that saquinavir and ritonavir have anti-tumour activities attributable to inhibition of the proteosome and induction of cell death [42,43]. The discrepancies with the reports mentioned earlier [9–11,18] might be explained by the doses used in these *in vitro* experiments.

Concentrations of indinavir and saquinavir in the plasma of HIV-infected individuals have been reported to range from 0.2 to 5.0 μM and from 0.1 to 4.0 μM, respectively [44]. Furthermore, drug concentrations are enhanced by the concomitant use of ritonavir, which facilitates absorption (via P-glycoprotein inhibition) and reduces clearance (via inhibition of cytochrome P450 3A-mediated metabolism) of saquinavir [45]. This therapeutic 'boosting' can result in plasma levels in excess of 7 μM. *In vitro* treatment with combined indinavir and saquinavir has an additive effect on cell death, suggesting additive effects on the mechanisms of the drug toxicity.

Molecular mechanisms involved in mitochondrion injury

How does genetic heterogeneity affect drug susceptibility and how do HIV drugs mediate damage to mitochondria? Cellular efflux pumps situated on the plasma membrane transport drugs out of the cell in an ATP-dependent fashion [46]. They were first identified in studies of cancer chemotherapy. The first of these ATP-binding cassette (ABC) transporters to be identified and characterized was P-glycoprotein (P-gp), the product of the human multidrug resistance gene *MDR1* [47]. More recently, these transporters have been categorized by the human gene nomenclature committee according to the ABC motif, with *MDR1* now being designated *ABCB1*. This family is now known to contain >50 distinct proteins, many of

which are as yet pharmacologically uncharacterized. However, many members of this transporter family have been shown to limit the bioavailability and/or cellular accumulation of HIV drugs. The multidrug resistance-associated proteins 1 and 2 [MRP-1 and MRP-2 (also known as ABCC1 and ABCC2, respectively)] transport several protease inhibitors [48], and MRP4 (also known as ABCC4) and MRP5 (also known as ABCC5) are nucleoside transporters that show affinity for NRTIs [49]. Lastly, the breast cancer resistance protein [BCRP (also known as ABCG2)] has been shown to limit the intracellular accumulation of NRTIs such as AZT [50].

Many of the aforementioned transporters have been shown to exhibit polymorphic expression. Although currently subject to substantial debate, the C3435T P-gp polymorphism has been associated with a greater $CD4^+$ T-cell increase during HAART, compared with patients with wild-type P-gp [51–55], and future studies will need to take individual drugs into consideration with carefully recruited patients [56]. However, of the other anti-retroviral-transporting proteins discussed above, genetic studies have not yet been conducted within the HIV arena.

Most of these transporters are functionally expressed at anatomical sanctuary sites for HIV-1 such as blood–tissue barriers (brain and testis) [57], macrophages and $CD4^+$ T cells [58], where they might influence the emergence of viral strains that escape effective chemotherapy. Indeed, it was shown recently that endogenous variation in the expression of P-gp was positively correlated with the EC_{50} of saquinavir in PBMCs [59] and these cells have at least some of the molecular machinery to induce P-gp expression in response to drugs and xenobiotics [60]. Thus, these transporters can extrude drugs and might limit the intracellular concentration of drugs (Figure 1) [61,62]. The HIV protease inhibitor ritonavir is a potent inhibitor of P-gp-mediated NRTI extrusion, and thereby increases the intracellular concentration of NRTIs [62]. Because the toxicity of NRTIs is cumulative, clinical thresholds for the concentration of NRTIs might be crucial in the acquired forms of mitochondrial illnesses that result from the toxicities associated with NRTIs and protease inhibitors.

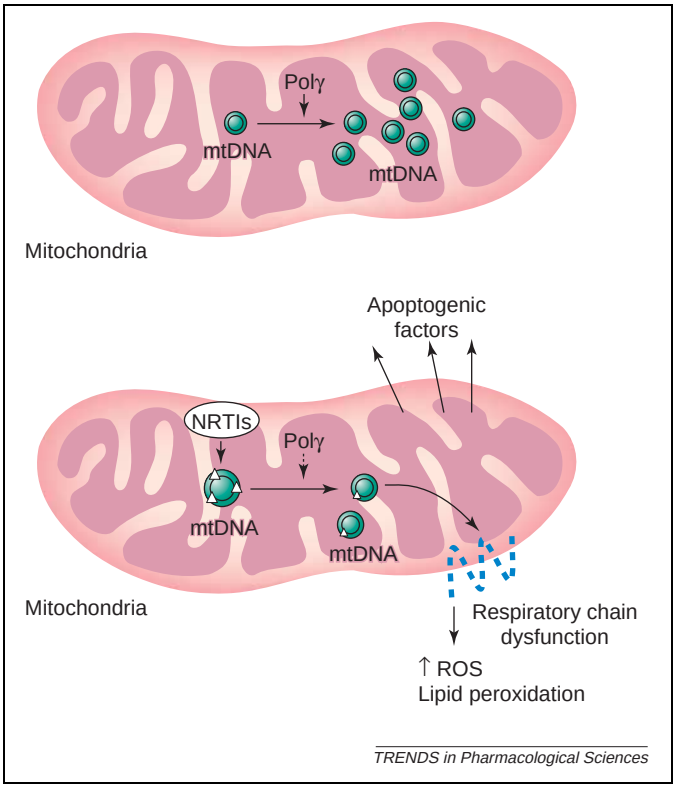

Figure 2. Inhibition of mitochondrial DNA (mtDNA) replication by anti-retroviral drugs, and subsequent mitochondriotoxic effects. DNA polymerase γ (Polγ) is required for the replication of mtDNA. Phosphorylated NRTIs compete with endogenous deoxyribonucleotides for incorporation into nascent DNA chains, thereby inhibiting Polγ. This leads to the depletion of mtDNA, dysfunction of the mitochondrial respiratory chain, increased generation of reactive oxygen species (ROS) and lipid peroxidation, and the release of apoptogenic factors.

To date, the mechanisms by which the HIV drugs induce mitochondrial dysfunction and cell death are not clearly established. Here, we present the putative molecular events involved in the mitochondriotoxic effects of both NRTIs and protease inhibitors.

Phosphorylated NRTIs compete with endogenous deoxyribonucleotides for incorporation into nascent DNA chains, and thereby inhibit DNA polymerase γ (Polγ) [5]. Polγ is required for the replication of mtDNA (Figure 2) and therefore inhibition of Polγ results in mtDNA depletion, altered mitochondrial oxidative phosphorylation enzyme activities and changes in mitochondrial morphology [63], thereby initiating the apoptotic cell death process [64]. NRTI-damaged cells with mitochondrial respiratory chain dysfunction (altered activity of oxidative phosphorylation enzymes, loss of cytochrome *c* oxidase, reduced mitochondrial ATP production and increased ROS levels) exhibit enhanced anaerobic ATP synthesis, leading to lactic acid production. This lactic acidosis is often a fatal outcome of extreme mitochondrial toxicity and can be an indicator that the drug should be discontinued. The generation of ROS and lipid peroxidation products impairs the respiratory chain, which results in a positive feedback effect generating even more ROS [65]. NRTIs induce cell death in many cell types [64,66,67] and therefore their utility as adjuvant anticancer therapies is being explored [66,67].

Insulin resistance is another commonly observed side-effect of HAART. Glucose uptake in skeletal muscle is reduced in treated patients and this might influence whole-body glucose disposal, which is reduced in patients receiving HAART [68,69]. The absence of glucose uptake following exposure to protease inhibitors might induce death through a process that is dependent on the mitochondria. Indeed, there is increasing evidence that cellular survival is related to the availability of growth factors that inhibit intrinsic programmed cell death, although severe restrictions in glycolysis result in cell death despite the continued presence of pro-survival growth factors. Surprisingly, death as a result of glucose limitation proceeds via an apoptotic mechanism. This is due to the activation of Bax, the release of cytochrome *c* and the activation of caspases [70,71]. In addition, Bax-dependent cell death can be induced by limiting the expression of the glucose transporter GLUT1 in murine blastocysts [72]. Looking specifically at glucose metabolism, subsequent investigations found that the ability of growth factors to maintain viability correlated with the

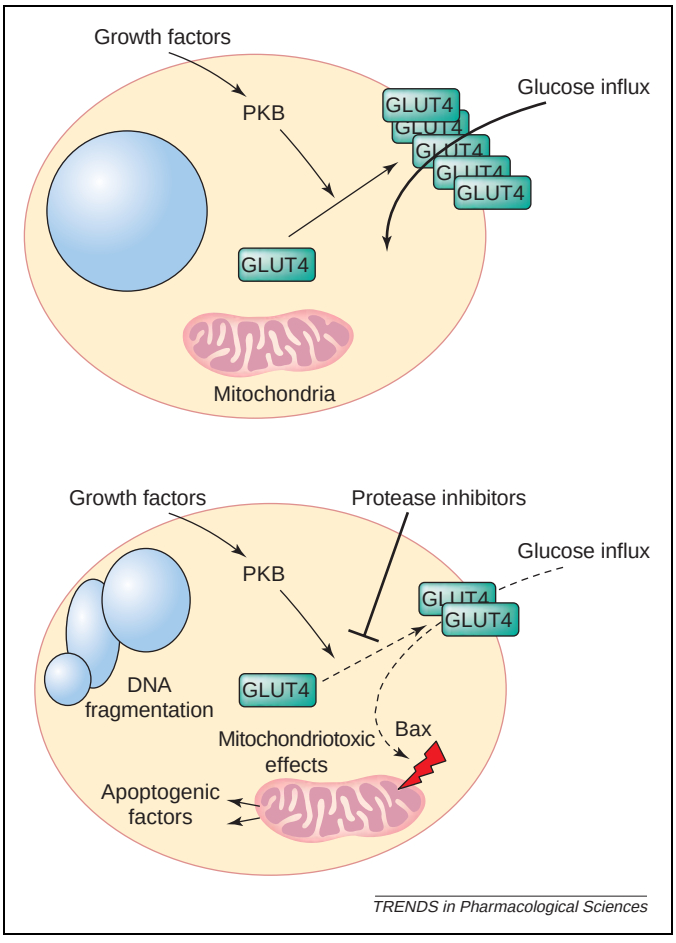

Figure 3. The effects of anti-retroviral drugs on the regulation of glucose uptake, and subsequent mitochondriotoxic effects. In normal conditions, growth factors activate protein kinase B (PKB), which stimulates glucose uptake through the translocation of the glutamate transporter GLUT4 to the plasma membrane. Protease inhibitors inhibit the translocation and/or intrinsic activity of GLUT4. The resulting decrease in glucose import results in augmented translocation of Bax and mitochondrial membrane permeabilization with the resultant release of apoptogenic factors.

expression of glucose transporters [73]. Protein kinase B (PKB), which is involved in cellular survival, has also been shown to mediate the effects of insulin on cellular metabolism [74]. In particular, PKB stimulates glucose uptake through the activation of GLUT1 and GLUT4 [75]. PKB can also induces GLUT4 translocation to the plasma membrane in adipocytes, increasing glucose uptake by these cells [76] (Figure 3). One of the first PKB targets identified was glycogen synthase kinase 3 (GSK-3), which inhibits glycogen synthase (and thus glycogen synthesis) in unstimulated cells [77]. PKB has been shown to phosphorylate (and therefore inhibit) GSK-3, enabling glycogen storage. Therefore, PKB could mediate cellular survival via mechanisms that partially involve activation of glycolytic metabolism. Several reports indicate that indinavir inhibits the translocation or intrinsic activity of GLUT4 [78,79]. Furthermore, prolonged exposure of adipocytes and skeletal muscle cells to nelfinavir decreases insulin-stimulated glucose transport and PKB phosphorylation [80]. However, a far more detrimental effect on the cell is seen when the glycolytic flux is reduced below a level needed to supply mitochondria with the substrates needed to maintain membrane potential and prevent cytochrome *c* release.

Concluding remarks

The development of HIV drugs and the great success in improving survival has been well documented. However, as discussed above the side-effects associated with the currently diverse therapies are multifactorial and the significant reduction of these toxicities in chronically exposed patients will be a significant challenge for the future.

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